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EFFECT OF DUCK HEPATITIS VIRUS ON PESTICIDE TOXICITY

W. L. Ragland

Georgia University

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Viral replication did not increase the activities of the microsomal enzymes and may have decreased the activities slightly. Viral replication prior to chemical induction of enzymes in chickens by phenobarbital and in ducks by DDT resulted in greater activities of the microsomal enzymes but not the soluble enzymes than was obtained by chemical induction without prior viral replication. Although the microsomal enzymes were induced to greater levels by viral replication, pesticide residue analysis failed to demonstrate increased clearance of residues from body tissues.

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By

W. L. Ragland
Poultry Disease Research Center
University of Georgia
953 College Station Road
Athens, Georgia 30601

Grant No. R801800

Project Officer

Dr. Ronald L. Baron
Environmental Toxicology Division
Health Effects Research Laboratory
Research Triangle Park, N.C. 27711

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
HEALTH EFFECTS RESEARCH LABORATORY
RESEARCH TRIANGLE PARK, N.C. 27711

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SECTION I

CONCLUSIONS

The replication of Newcastle disease vaccinal virus in the livers of chickens and the duck hepatitis virus in the livers of adult ducks facilitates the induction of hepatic microsomal enzymes by subsequent chemical induction. The increased levels of activity do not persist and appear to be modulated by other factors such as the viral replicatory cycle. Increased clearance of pesticide from body tissues could not be demonstrated in virus-treated birds even though the microsomal drug-metabolizing enzymes were induced. Although replication of duck hepatitis virus in adult ducks causes specific changes in the hepatic, microsomal, drug-metabolizing enzymes, the results of the present investigation do not support the theory that these changes are responsible for the protection against pesticide toxicity provided by duck hepatitis virus.

SECTION II

RECOMMENDATIONS

The specific interrelationships between viral replication on hepatic endoplasmic reticulum and the drug-metabolizing enzymes of the hepatic endoplasmic reticulum should continue to be investigated. Whether these phenomena are of major importance from the point of view of environmental protection or of more importance from a clinical health services point of view must be evaluated by the appropriate governmental agencies. They certainly are of considerable biological importance and should be investigated for the basic information to be gained if not for any other reason.

The effect of exposure to xenobiotic compounds on subsequent viral replication should be investigated in addition to the effect of viral replication on induction of the drug-metabolizing enzymes.

In spite of the study reported here and the reports of others in the literature, a good experimental model system for the study of the interrelationships between microsomal drug-metabolizing enzymes and viral replication is still lacking. A good experimental model must first be found and characterized before any substantial progress can be made in this interesting area of research.

SECTION III

INTRODUCTION

GENERAL

Xenobiotic compounds such as drugs, chemical pesticides and industrial pollutants will induce elevated activities of a number of enzymes collectively known as the drug-metabolizing enzymes. Since the first description of drug-metabolizing enzyme activity by Mueller and Miller[1] a great deal of effort has been devoted to these enzymes and their inducers. So much attention has been given to the chemical inducers that it has been assumed by many investigators that chemical agents are the only significant modulators of drug-metabolizing enzyme induction. Nevertheless, it is becoming increasingly evident that profound interrelationships exist between certain viruses and the hepatic enzymes which metabolize drugs and pesticides. It is obvious that any infectious disease which causes frank hepatic necrosis or severe cachexia will alter drug metabolism. There are scattered in the literature, however, reports of specific effects of viral replication on drug metabolism. The main characteristics which these viruses have in common are (1) they are RNA viruses and (2) they replicate and/or are assembled on hepatic smooth endoplasmic reticulum. Very pronounced morphologic changes are produced by some of these viruses in the endoplasmic reticulum[2] and, hence, it seems plausible, even likely, that significant functional alterations would result from the morphologic changes. The published reports of viruses affecting hepatic drug-mebabolism have been summarized below.

Murine Hepatitis

Kato et al.[3] reported that murine hepatitis viruses in Swiss mice caused initially a stimulation of hexobarbital and strychnine metabolism

which was followed by a steady decline in activity. Approximately 12 hours after infection the metabolism of these drugs had been increased 25% and 24 hours after infection the activity had dropped to control levels. There was a steady decline in activity and 72 hours following infection the metabolism of these drugs had been decreased 50% below control levels. Beyond this point there was a steady increase and return to normal levels.

Duck Hepatitis

Friend [4] discovered that injection of adult mallards with duck hepatitis virus increased resistance to DDT and dieldrin. Not only was the number of deaths from pesticide toxicity decreased but the length of time on the contaminated feed required to produce deaths was prolonged and deaths were observed only during the last several days of the experiment. Had the ducks been kept on the feed regimen longer, the mortality curve may have been identical to controls, only delayed in its appearance. Considering the possibility that an interaction between viral replication and the pesticide-metabolizing enzymes of the hepatic smooth endoplasmic reticulum might be the basis for the protection, Ragland et al. [5] determined the effect of duck hepatitis virus replication in adult ducks on ethylmorphine N-demethylation by postmitochondrial supernatant fractions of liver. Mallard drakes were inoculated with duck hepatitis virus 48 hrs prior to exposure to DDT in the diet for 7 days. At 7 days the level of enzyme activity was lower in birds inoculated with the virus and fed a normal diet than in normal birds fed control diet. Enzyme activity in non-virus-treated birds fed DDT was induced about 4 x over normal birds fed a normal diet. Enzyme activity in the virus-treated birds fed DDT was about 22% (based on per gm liver) to 37% (based on per total liver) higher than in the non-virus-treated birds fed DDT.

Infectious Hepatitis B of Humans

Doshi et al. [6] obtained liver biopsy material from patients in the necrotic and regenerative phases of infectious hepatitis and measured several parameters, one of which was pentobarbital hydroxylase. The authors found that whereas pentobarbital hydroxylase activity was markedly reduced during the necrotic phase of hepatitis it was greatly increased during the regenerative phase. The decrease in the necrotic phase was

understandable and had been anticipated but the increased activity which followed was considered noteworthy as most investigators have found that drug-metabolizing enzymes cannot be induced with chemicals during hepatic regeneration. In speculating on the cause of the unexpected stimulation of enzyme activity the authors mentioned the possibility of a specific effect of viral replication. They further speculated that the elevated enzyme activity may explain the difficulty experienced in the sedation of convalescent hepatitis patients with barbiturates.

JUSTIFICATION AND OBJECTIVE OF THE PRESENT STUDY

The observations are significant in that they indicate that chemicals may not be the only important environmental contaminants which alter drug-metabolism. As our primary concern in infectious diseases has shifted from acute and chronic infectious disease to attenuated infections (masked, latent, inapparent infectious, microbial persistence, etc.), we have become involved with the subtle, long-term effects (such as oncogenesis) of infectious agents which do not produce overt infectious diseases. Such conditions are known to occur. Persistent viral hepatitis in man is not an inconsequential matter. Another interesting example is the Ulster strain of Newcastle disease virus. Ireland has remained free of Newcastle disease for years, but when serologic surveillance of Irish birds was instituted, it was learned that although Ireland was free of the disease, it was not free of the virus. The apparent paradox was solved by the isolation and characterization of the Ulster strain which is a non-pathogenic but immunogenic strain of Newcastle disease virus whose existance had never been even suspected. In this report we will demonstrate that vaccinal Newcastle disease virus is another virus which alters hepatic drug-metabolism. There may be additional viruses yet undetected which may have similar effects in various species, including man. But it is not necessary to conjure up undiscovered viruses. Most of the live virus vaccines, including human vaccines, utilize RNA viruses which replicate on hepatic smooth endoplasmic reticulum, and they may be affecting the hepatic drug-metabolizing enzymes. The assumption that there are viruses which do affect hepatic drug-metabolism in man, and even evidence that hepatitis type B virus does, the mechanisms

involved need to be ascertained in order that the significance of their interaction with other non-infectious, environmental contaminants, i.e. chemicals, can be evaluated.

Thus, the present study was undertaken to examine further the interrelationship between duck hepatitis virus and pesticide toxicity. It was necessary to modify the research as the project was funded for only one year instead of the three years as proposed. The effects of viral replication of duck hepatitis virus in adult mallard drakes on several hepatic drug-metabolizing enzyme activities and on tissue residues of DDT were evaluated. The protocol was changed to include an investigation into the effects of Newcastle vaccine virus replication on enzyme activities of chickens since it is not a virus with preferential replication in liver and, if the results were positive, they would provide evidence that specific effects of viral replication on drug-metabolism is not confined to a small group of hepatitis viruses.

SECTION IV

ANALYTICAL METHODS

PREPARATION OF POSTMITOCHONDRIAL SUPERNATANT AND MICROSOMAL FRACTIONS OF AVIAN LIVER.

All birds were killed by decapitation using a guillotine. Livers were excised free of adhering tissue, rinsed, blotted and weighed. All tissue preparations were maintained at 0-4 C. Liver weights were recorded and 10 g portions taken for homogenization in 30 ml 0.15 M KCl using ten strokes of a motor-driven teflon pestle in a Potter-Elvehjem homogenizer. The resulting 25% (w/v) homogenates were centrifuged in a Sorval SS-34 or Beckman JA.. rotor for 20 min at 10,000 x g max. The postmitochondrial supernatant fractions were decanted and aliquots saved for assay of nitroreductase activity.

The greater portion of the postmitochondrial supernatant fraction was spun for 20 min at 346,000 x g max in the Type 65 rotor of a Beckman L2-65B ultracentrifuge. The clear supernatant fractions were discarded and the microsomal pellets suspended in 0.15 M KCl to the volumes of the postmitochondrial supernatant fractions from which they were derived. One milliliter of microsomal suspension usually contained 4-6 mg protein (assayed by the method of Lowry, et al.[7] using bovine serum albumin, fraction V, as standard) and represented 300-350 mg fresh liver.

Enzyme Assays

Procedures were derived for assaying the activities of several drug-metabolizing enzymes of avian liver. They were based on procedures used for mammalian systems but modified to assure substrate excess and first order kinetics with time and enzyme concentration. All assays of

drug-metabolizing enzymes were carried out in 25 ml Erlenmeyer flasks in a metabolic shaker bath maintained at 37 C. Except as noted, reaction vessels were shaken at 120 cycles per min under air.

Ethylmorphine N-Demethylase

The N-demethylation of ethylmorphine by 1.0 ml of post mitochondrial supernatant fractions or 1.0 ml of microsomes was assayed according to the method of Sladek and Mannering.[8] The formaldehyde produced was measured by a modified method of Nash[9] as described by Anders and Mannering,[10] except that a 5.5% rather than a 5.0% $ZnSO_4 \cdot 7H_2O$ solution was employed. First order kinetics were observed with time (Fig. 1) and enzyme concentration (Fig. 2).

Aryl Hydrocarbon Hydroxylase

The assay procedure for the determination of aryl hydrocarbon (benzo-(a)pyrene) hydroxylase activity was essentially that of Nebert and Gelboin.[11] Adjustments were made for a total reaction volume of 3 ml, and postmitochondrial supernatant fractions or microsomes were used instead of the cell homogenate. In addition, bovine serum albumin, fraction V, (final concentration, 1 mg/ml) was included to solubilize the substrate (Alvares et al.).[12] When enzyme activities were assayed with fractions from chicken liver, 0.3 ml of enzyme was used and the reaction time was 15 min. First order kinetics with time and enzyme concentration are demonstrated in Figs. 3 and 4. The amount of enzyme was reduced to 0.1 ml microsomes and the reaction time was shortened to 5 min when assays were performed with duck liver fractions in order to assure first order kinetcs (Figs. 5, 6, and 7). Reactions were stopped with 3 ml cold acetone. Ten milliliters of hexane were added and the mixtures incubated an additional 10 min with shaking. A 2 ml sample of the organic phase was extracted with 6 ml 1N NaOH and the products assayed in a Turner model 111 spectrofluorometer equipped with filters selected for activation (Wratten 47B, maximum transmittance at 430 nm) and fluorescence (Wratten 58, maximum transmittance at 530 nm). Since several related products are measured in this assay, the enzyme activities were recorded in arbitrary fluorescence units rather than using the 8-hydroxy derivative as a standard.[13] This method of

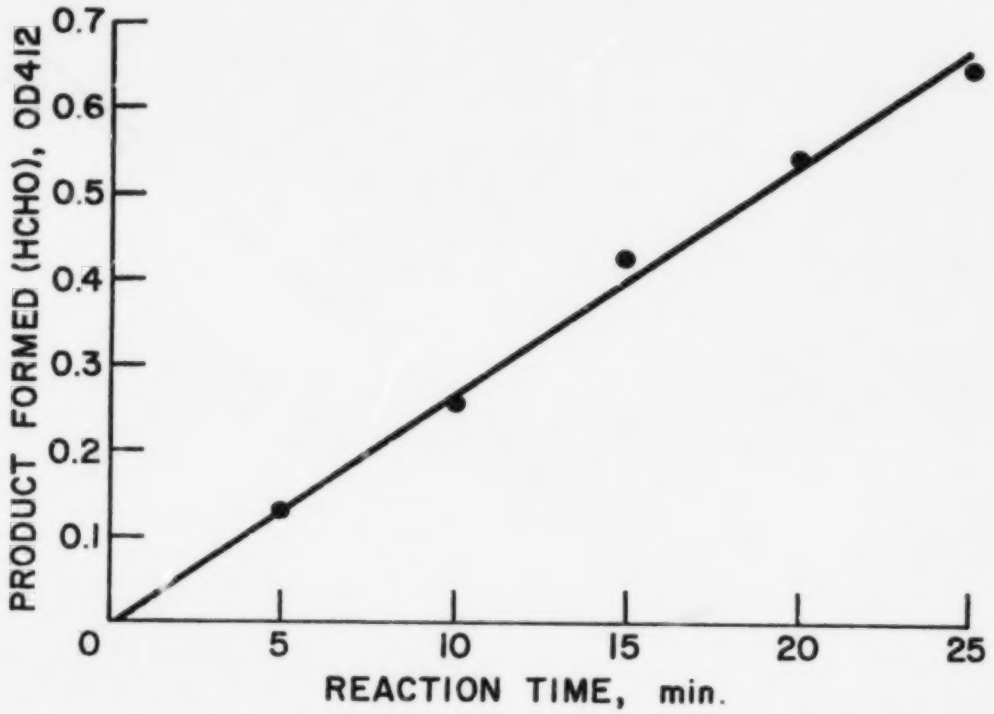

Figure 1. First order kinetics with time of N-demethylation of ethylmorphine by 1.0 ml of liver microsomes from chickens induced with phenobarbital.

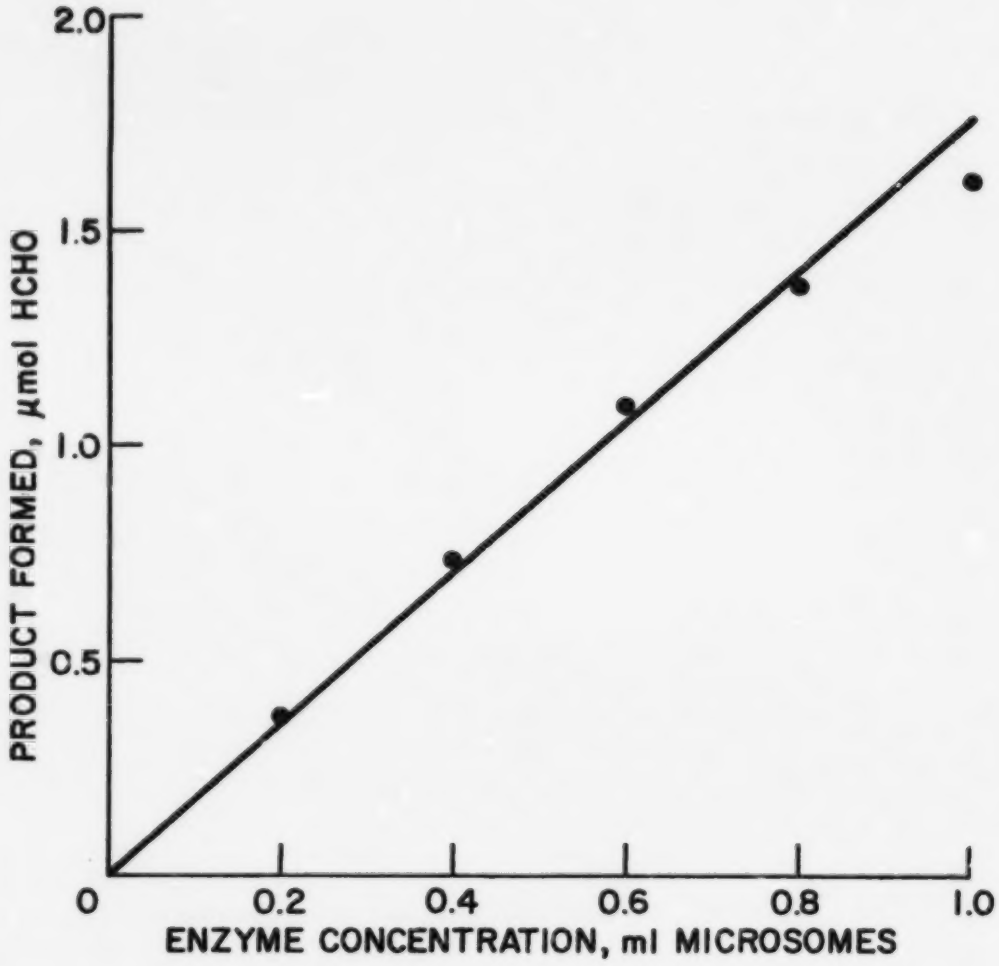

Figure 2. First order kinetics with enzyme concentration of N-demethylation of ethylmorphine in 15 min reaction. Liver microsomes were prepared from chickens induced with phenobarbital.

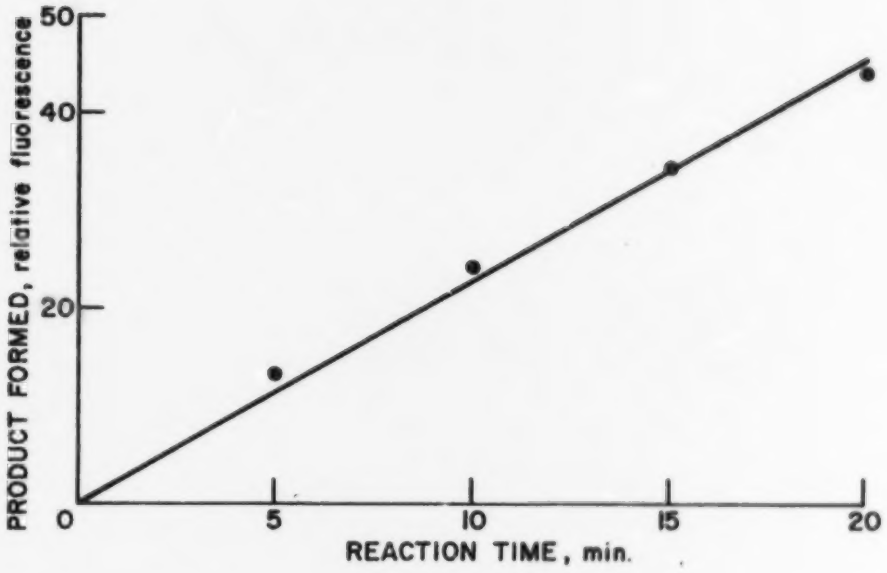

Figure 3. First order kinetics with time of aryl hydrocarbon hydroxylation of benzo(a)pyrene by 0.3 ml of liver postmitochondrial supernatant fraction from chickens induced with 3-methyl-cholanthrene. A NADPH generating system was used.

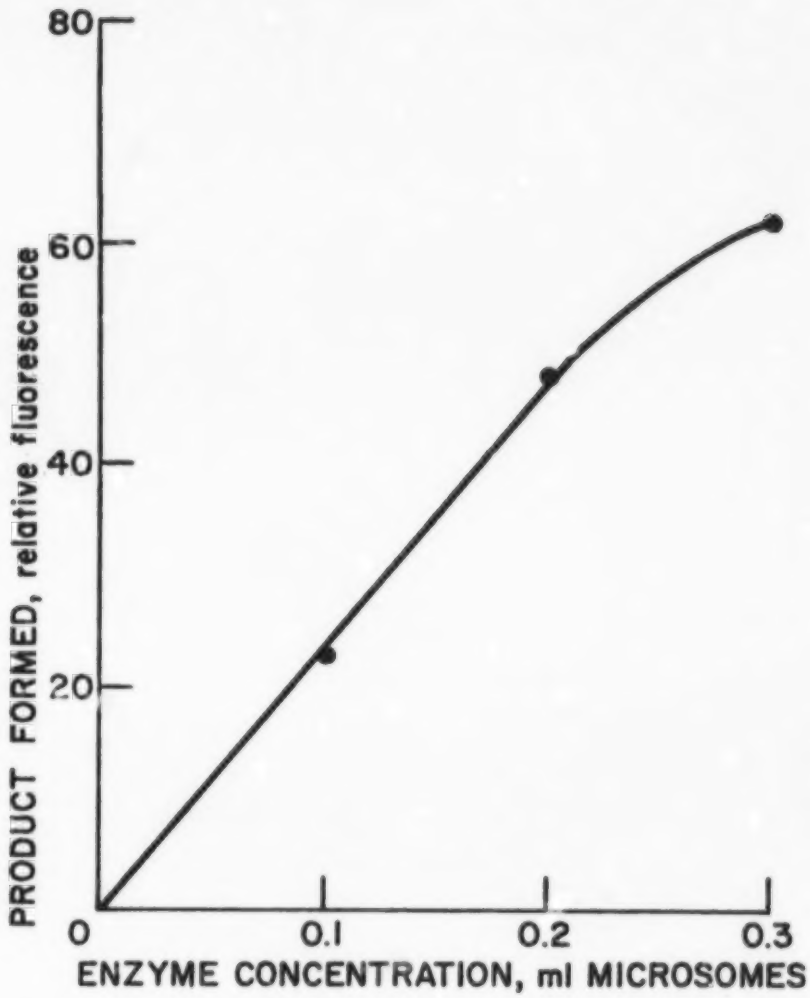

Figure 4. First order kinetics with enzyme concentration of the aryl hydrocarbon hydroxylation of benzo(a)pyrene in 15 min reaction using NADPH generating system. Liver microsomes were prepared from chickens induced with phenobarbital.

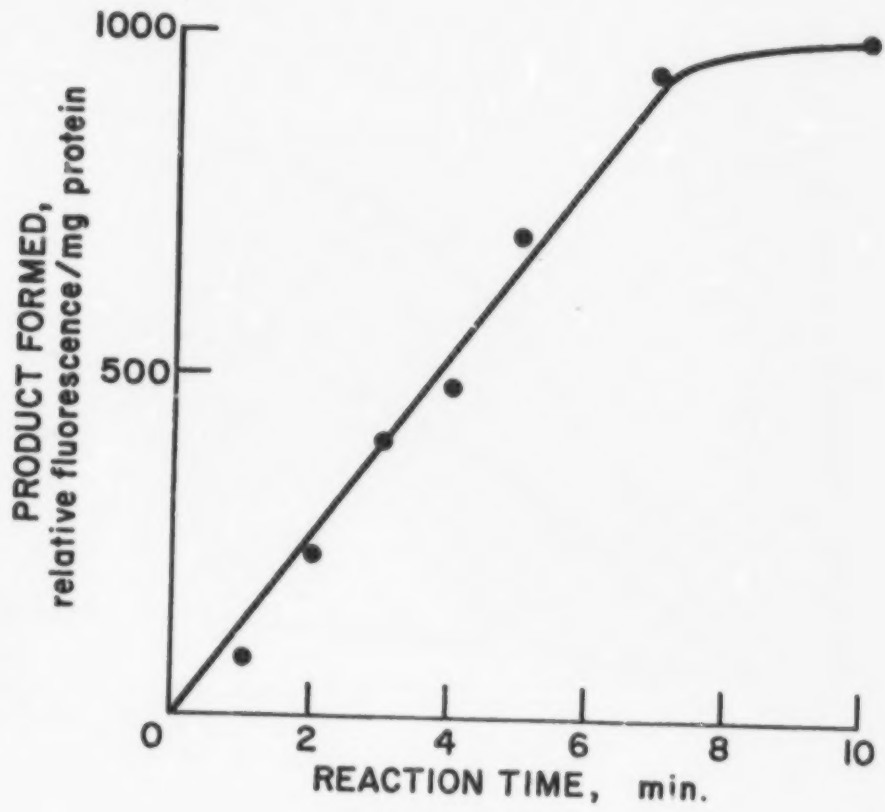

Figure 5. First order kinetics with time of aryl hydrocarbon hydroxylation of benzo(a)pyrene by 0.1 ml of liver microsomes from ducks induced with phenobarbital. NADPH was added to the reaction mixture.

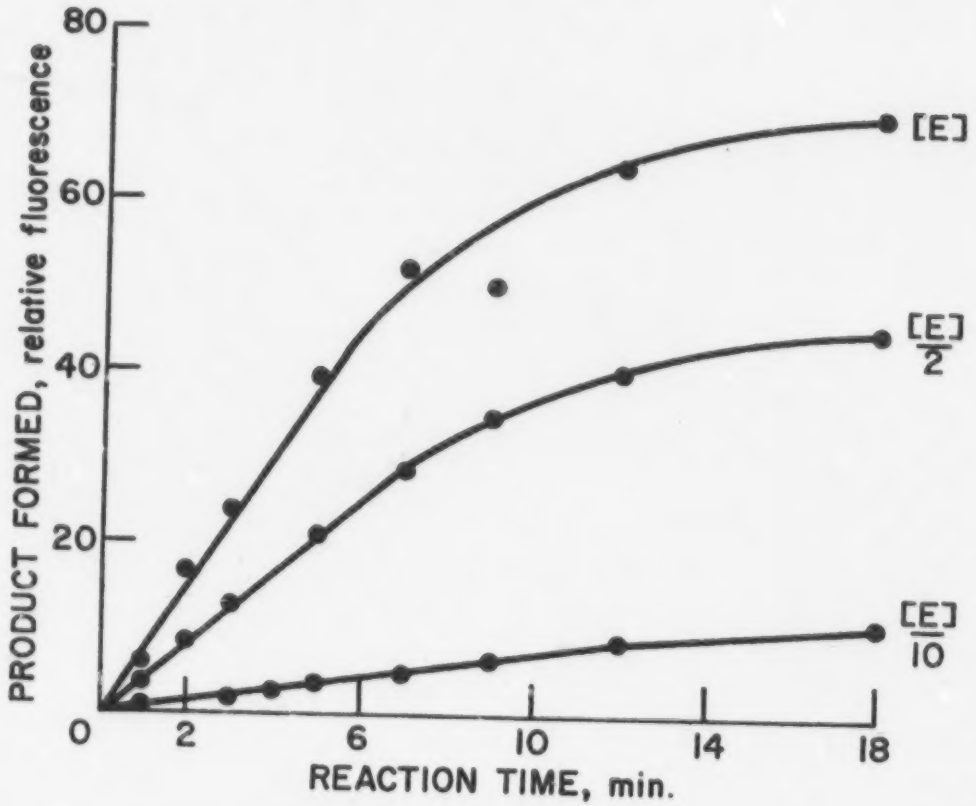

Figure 6. First order kinetics with time of aryl hydrocarbon hydroxylation of benzo(a)pyrene by 0.1 ml of liver microsomes from ducks induced with 3-methyl-cholanthrene. First order kinetics were obtained with 2 and 10 fold dilutions of microsomes. First order kinetics with enzyme concentration is also demonstrated by amount of product formed for each of the three enzyme concentrations assayed. A NADPH generating system was used.

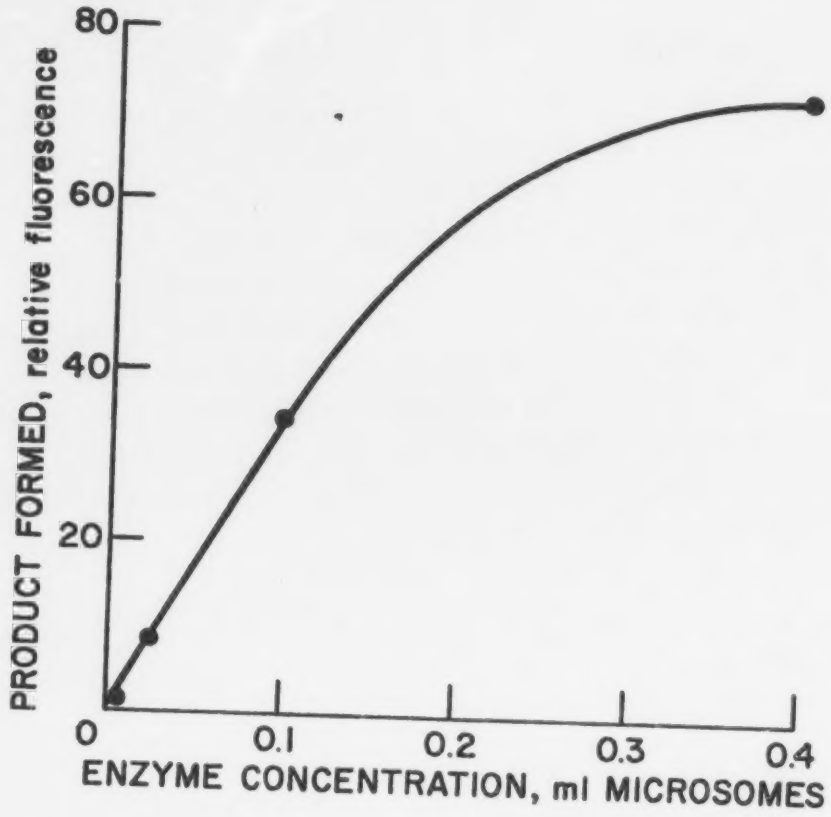

Figure 7. First order kinetics with enzyme concentration of the aryl hydrocarbon hydroxylation of benzo(a)pyrene in 5 min reaction using NADPH. Liver microsomes were prepared from ducks induced with phenobarbital.

recording the enzyme activity has been discussed and justified by Hansen and Fouts.[14]

Aniline Hydroxylase

The reaction mixture used in the assay of aniline hydroxylase activity contained, in a total of 5 ml, 50 mM phosphate buffer, pH 7.4, 10 mM aniline (added in 0.1 ml acetone), 0.4 mM NADP, 4 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$ and 1 ml microsomes. The reactions were incubated for 20 min, stopped with 2.5 ml 20% TCA and centrifuged to remove precipitated protein. The p-aminophenol produced in the reaction was assayed by the method of Imai and Omura[15] as follows: 1 ml of clarified reaction mixture was mixed with 1 ml 1% phenol in 0.5 M NaOH and 1 ml 1 M Na_2CO_3. After 20 min the product was assayed spectrophotometrically at 620 nm. First order kinetics with time and enzyme concentration are demonstrated in Figs. 8 and 9.

Nitroreductase

The reaction mixture used for assay of nitroreductase consisted of the following: 100 mM phosphate buffer, pH 7.4, 2 mM p-nitrobenzoic acid, 0.2 mM NADPH, 0.5 mM FMN, 5mM each of cysteine, niacinamide and $MgCl_2$, and 1 ml enzyme postmitochondrial supernatant fraction in a total volume of 5 ml. The reaction flasks were fitted with sidearms which held the enzyme until the reactions were initiated. The flasks were equilibrated to 37 C, gassed with N_2, tightly capped, and tipped to mix the contents. They were shaken at 60 cycles per minute for 30 min. Reactions were stopped with 5 ml cold 10% TCA and centrifuged to remove the precipitated protein. The p-aminobenzoic acid produced in the reaction was determined by the method of Bratton and Marshall[16] as modified by Juchau[17] except that absorbance was read at 545 nm as specified by the original authors. First order kinetics with time and enzyme concentration are demonstrated in Figs. 10 and 11.

PESTICIDE RESIDUE ANALYSIS

Extraction Procedures

Fat tissue - Samples of abdominal fat, taken from chickens adulterated with dieldrin, were prepared for gas-liquid chromatography as follows:

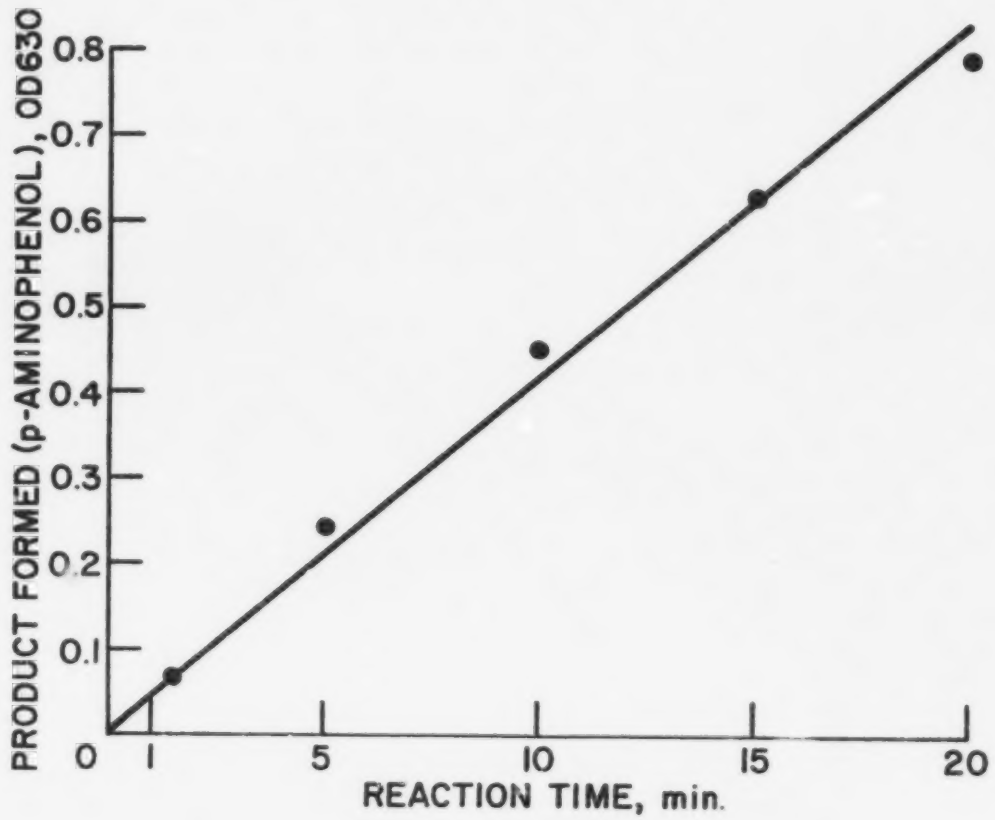

Figure 8. First order kinetics with time of aniline hydroxylation by 1.0 ml of liver microsomes from chickens induced with phenobarbital.

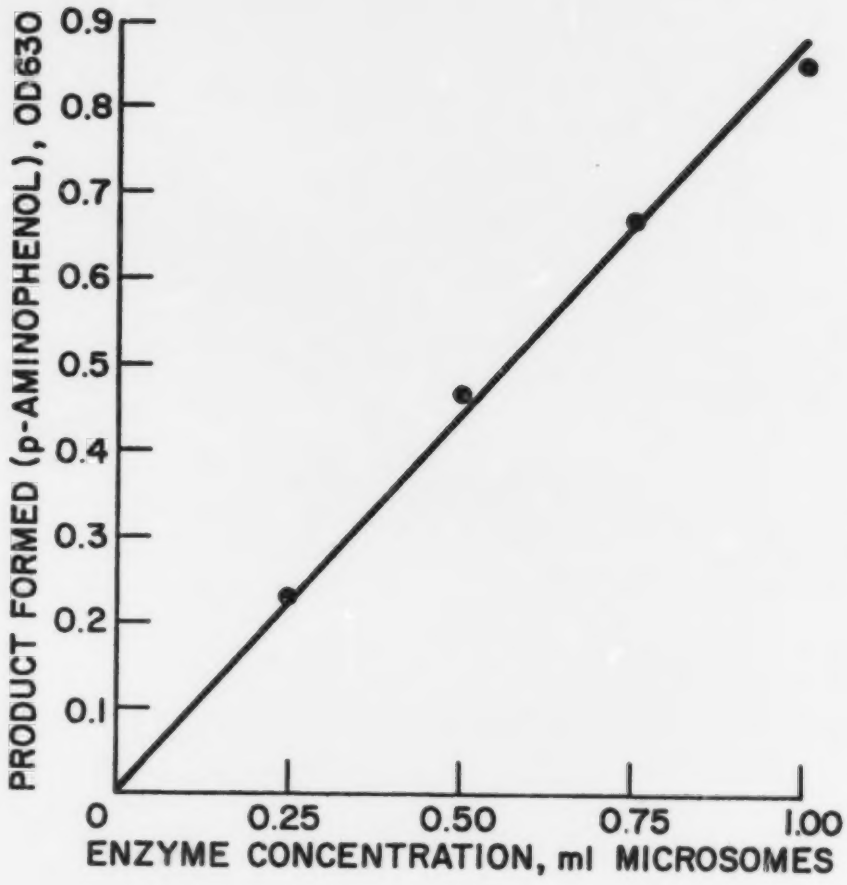

Figure 9. First order kinetics with enzyme concentration of aniline hydroxylation in 20 min reaction. Liver microsomes were prepared from chickens induced with phenobarbital.

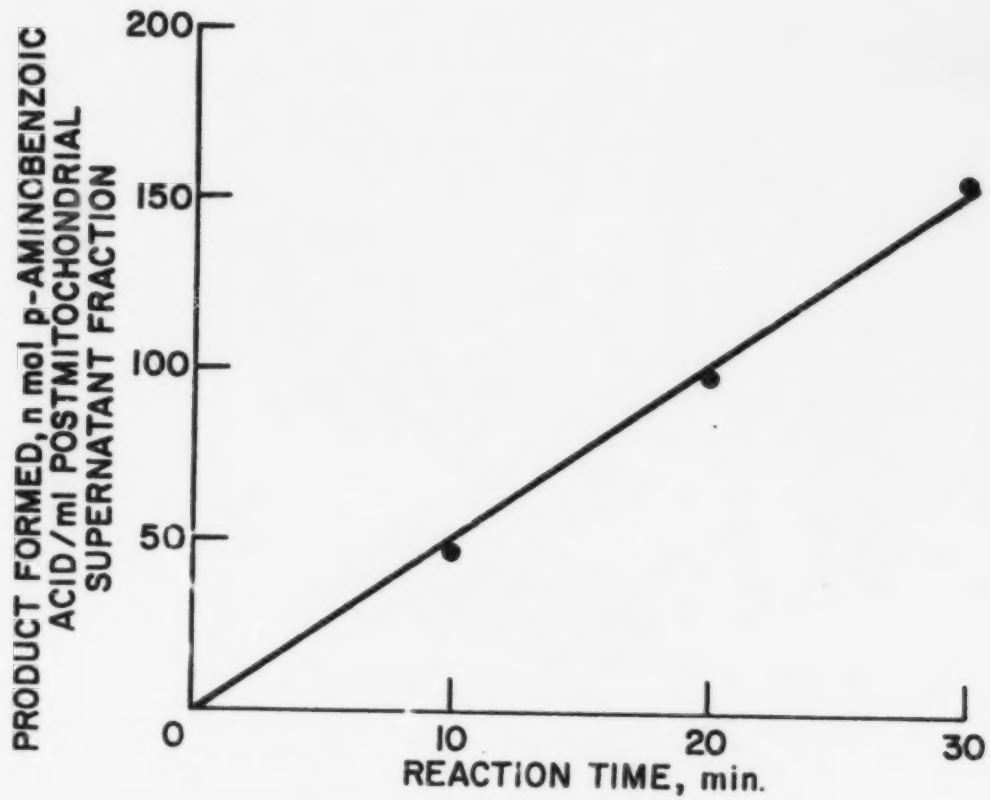

Figure 10. First order kinetics with time of nitroreduction of p-nitrobenzoic acid by 1.0 ml of liver postmitochondrial supernatant fraction from chickens induced with phenobarbital.

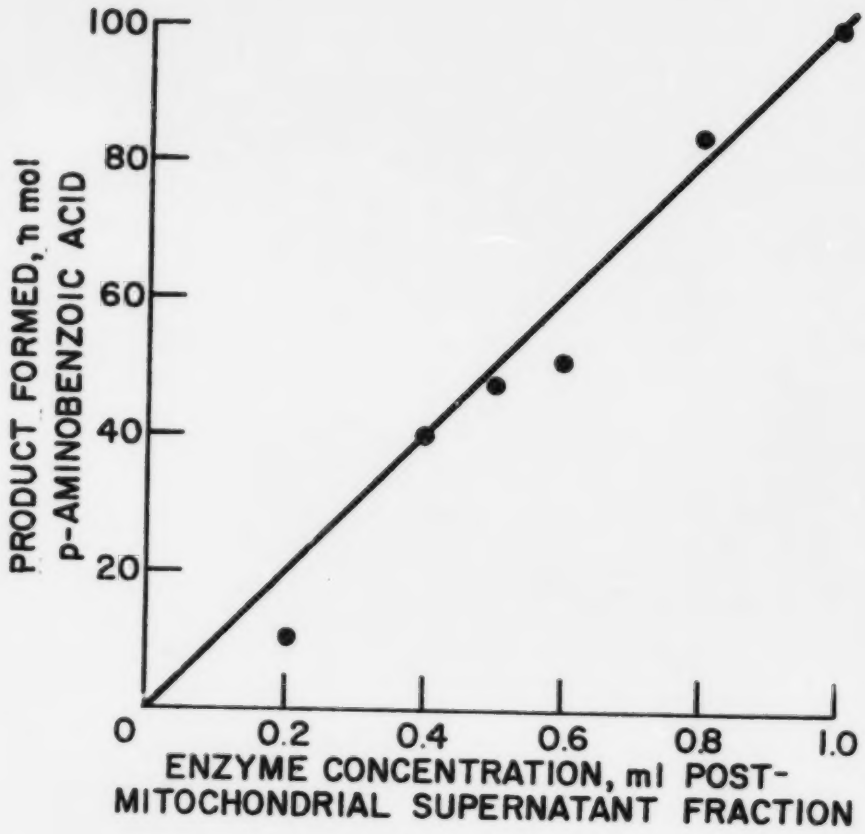

Figure 11. First order kinetics with enzyme concentration of p-nitrobenzoic acid in 20 min reaction. Liver postmitochondrial supernatant fractions prepared from chickens induced with phenobarbital.

1 g samples were macerated to a dry powder with 2 g anhydrous Na_2SO_4. The mixture was extracted with successive 15 ml portions of hexane by vigorous mixing in a 45 ml centrifuge tube for 60 sec on a vortex mixer. The combined extracts were concentrated to 20 ml by evaporation and cleaned up by the acetonitrile partitioning method described by Jones and Reddick[18] as modified by Mills[19] and further refined by Samuel.[20] Volumes were scaled down approximately and pure hexane replaced pet ether. The final sample in washed hexane was evaporated to dryness and the residue taken up in 10 ml hexane for chromatographic analysis.

Serum - DDT and its metabolites DDD and DDE were extracted from blood serum of exsanguinated mallards by two methods. The first was a simplification of Dale's procedure.[21] It involved the extraction of 1 ml serum with 5 ml hexane in a centrifuge tube vortexed rapidly for 3 min. The hexane phase was sampled directly after 5 min centrifugation at 800 x g max.

The second extraction method, which gave somewhat higher recoveries, was adapted for pesticides from the lipid extraction procedure of Bligh and Dyer.[22] Samples of 1 ml serum was extracted with 5 ml 1:1 chloroform-methanol by rapid vortexing for 60 sec. Ten milliliters distilled water were added and the mixture vortexed an additional 10 sec. After centrifugation for 15 min, 100 µl of the chloroform layer were removed, evaporated to dryness, and reconstituted with hexane.

Brain - Samples of 0.5 g duck brain were macerated to dryness with 1 g Na_2SO_4 in a watch glass. Further extraction was done as with the serum samples.

Bile - The lipid extraction method of Folch[23] using 2:1 chloroform-methanol was adapted to small samples of bile. Samples of 200 µl duck bile containing DDT were combined with 4 ml $CHCl_3$ and 2 ml MeOH in 45 ml centrifuge tubes and vortexed rapidly for 2 min. Ten milliliters water were added and the vortexing continued for 15 sec. Centrifugation for 5 min at 800 x g max. separated the two phases, and aliquots of the chloroform layer were treated as described above

Chromatographic Technique

The gas chromatograph was a Tracor model 222. It was equipped with a ^{63}Ni electron capture detector, a linearizer, and a Linear Instruments

model 252 integrating recorder. Columns were of glass, U-shaped, 1.8 m x 3.5 mm i.d. The carrier gas was 95% argon/5% methane at 40 p.s.i. and passed through a molecular sieve drier cartridge. All samples were injected in 5 µl pesticide grade hexane.

The column used for the assay of dieldrin residues was packed with 1.5% OV-17, 1.95% QF-1 on 100-120 mesh chromosorb W. Carrier flow was 75 ml/min with 25 ml/min purge. Column oven, inlet and detector temperatures were 225 C, 240 C, and 300 C respectively. A 5% OV-1 column was used intermittently for verification of peak indentities.

The column used for DDT and its metabolites contained 1.5% OV-1, 2.5% QF-1 on 100-120 mesh chromosorb W, H.P., acid-washed and DMCS-treated. Oven, inlet and detector temperature were maintained at 195 C, 220 C and 250 C, respectively. Carrier flow was routinely 70/30.

The percent recovery of each pesticide or metabolite was determined individually by quantifying recoveries of known amounts of standards after extraction from the corresponding body fraction of an untreated control. All pesticide standards were obtained from the U. S. Environmental Protection Agency, Pesticides and Toxic Effects Laboratory, Research Triangle Park, N. C. The degree of purity of each standard was established in our laboratory before use.

SECTION V

EFFECT OF NEWCASTLE DISEASE VACCINAL VIRUS ON THE INDUCTION OF DRUG-METABOLIZING ENZYMES IN CHICKEN LIVER.

One-day-old broiler type chicks which had not been treated with any vaccine or drug were housed in modified Horsfall-Bauer units and maintained in filtered air under positive pressure. They were fed growing ration feed containing no chemical additives and had free access to water.

When the birds were 8 weeks old they were vaccinated intraperitoneally with 5×10^6 chick embryo LD_{50} of the LaSota B-1 strain of Newcastle disease virus per kilogram body weight or equivalent volumes of sterile diluent. Three days later the birds were given intraperitoneally 40 mg sodium phenobarbital per kg body weight or equivalent volumes of sterile saline daily for 3 days. The birds were killed on the 7th day by decapitation and hepatic microsomes were prepared for assay of the 3 microsomal enzymes, N-demethylase, aryl hydrocarbon hydroxylase and aniline hydroxylase. Aliquots of the postmitochondrial supernatant fraction were used to assay the soluble enzyme, nitroreductase.

The enzyme activities observed in pullets are given in Figs. 12-15 and the activities in cockrels are given in Figs. 16-19. The levels of the drug-metabolizing enzymes in vaccinated birds were not markedly different from the levels in the unvaccinated saline-treated controls. Activities of the 3 microsomal enzymes were elevated considerably ("induced") by treatment with phenobarbital. On the other hand, nitroreductase activity in the postmitochondrial supernatant fraction was not elevated by phenobarbital in non-vaccinated birds. The activities of the microsomal enzymes were considerably greater in the vaccinated birds given phenobarbital than in the non-vaccinated birds given phenobarbital. Ethylmorphine

N-demethylase was 84% higher in pullets and 41% higher in cockrels. Aryl hydrocarbon hydroxylase was 51% greater in pullets ($p < 0.05$) and 153% greater in cockrels ($p < 0.001$). Aniline was 39% greater in pullets and 18% greater in cockrels. On the other hand, the activity of nitroreductase was 44% lower in pullets ($p < 0.05$) and 20% lower in cockrels.

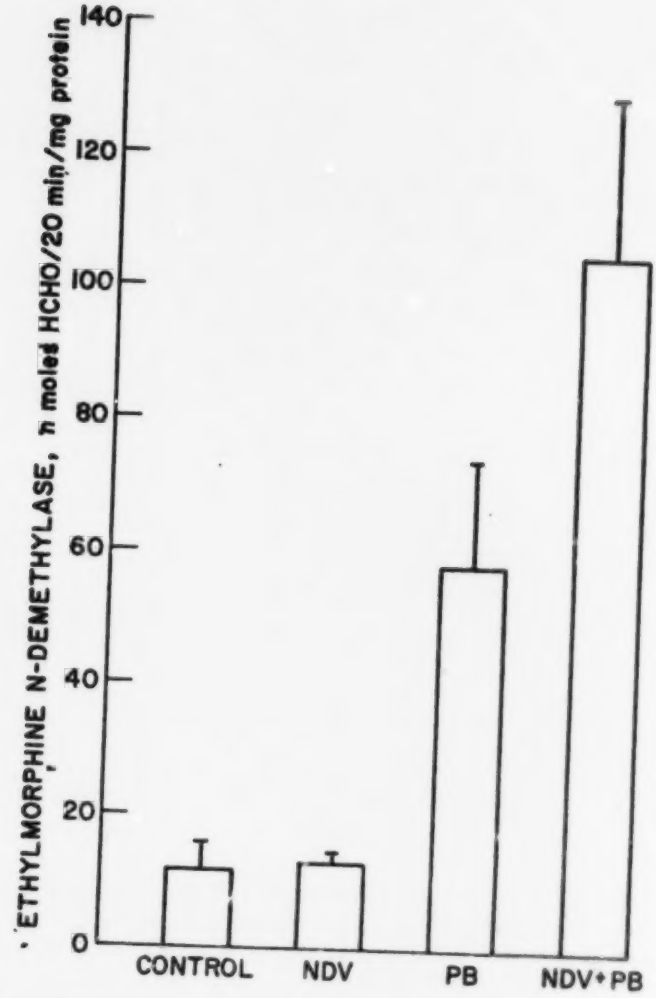

Figure 12. Effect of Newcastle disease vaccination on hepatic microsomal ethylmorphine N-demethylase of eight-week-old pullets. NDV = Newcastle disease vaccinated group. PR = phenobarbital induced group. NDV + PB = group vaccinated with Newcastle disease virus and subsequently induced with phenobarbital. Data are mean activities ± std error of the mean for 5 chickens.

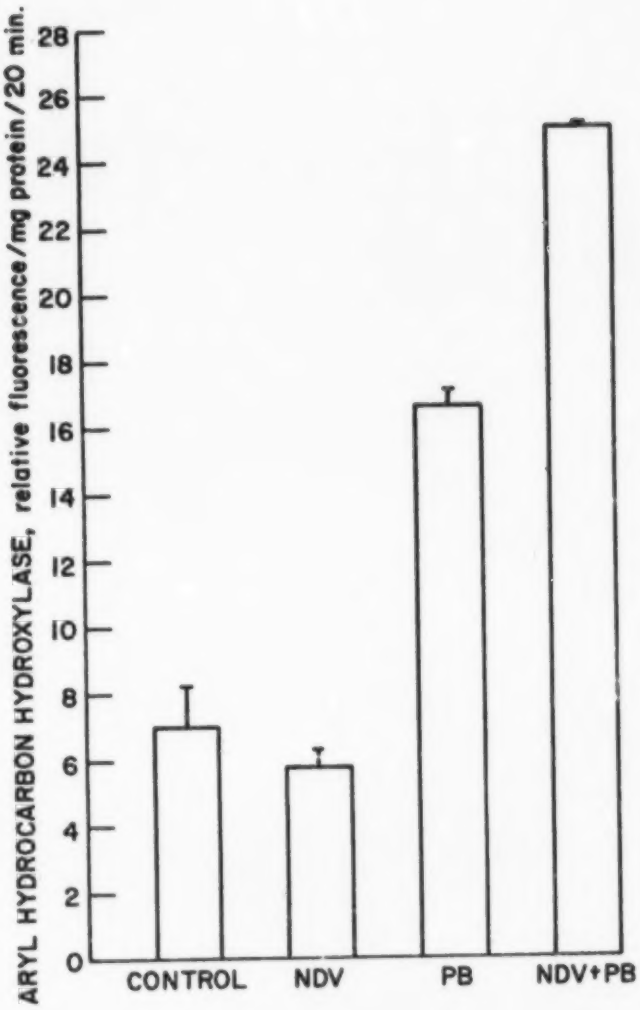

Figure 13. Effect of Newcastle disease vaccination on hepatic microsomal aryl hydrocarbon hydroxylase of eight-week-old pullets. NDV = Newcastle disease vaccinated group. PB = phenobarbital induced group. NDV + PB = group vaccinated with Newcastle disease virus and subsequently induced with phenobarbital. Data are mean activities ± std error of the mean for 5 chickens.

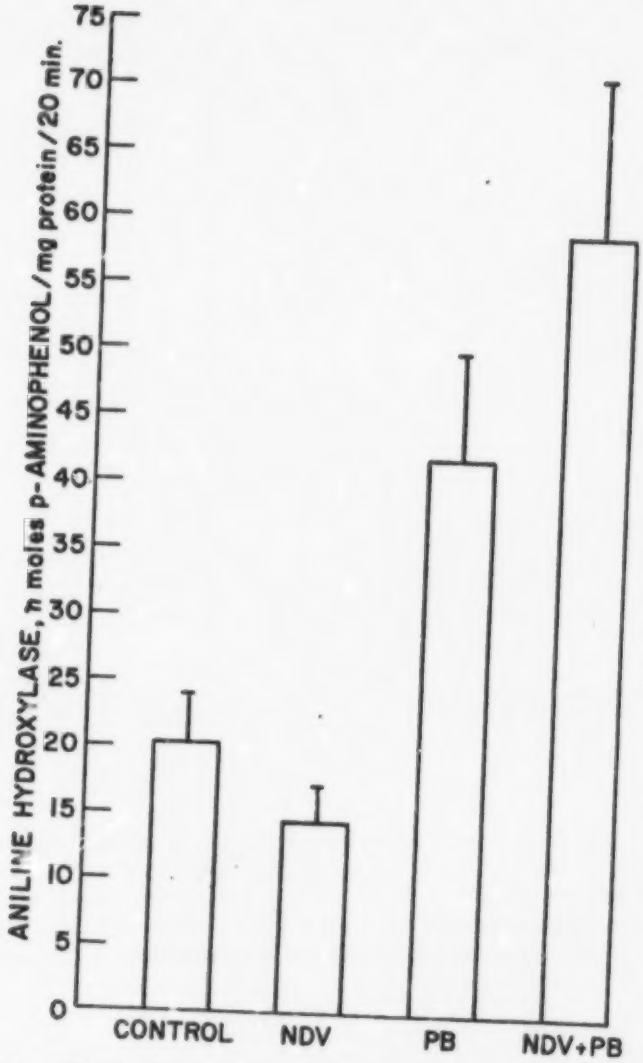

Figure 14. Effect of Newcastle disease vaccination on hepatic microsomal aniline hydroxylase of eight-week-old pullets. NDV = Newcastle disease vaccinated group. PB = phenobarbital induced group. NDV + PB = group vaccinated with Newcastle disease virus and subsequently induced with phenobarbital. Data are mean activities ± std error of the mean for 5 chickens.

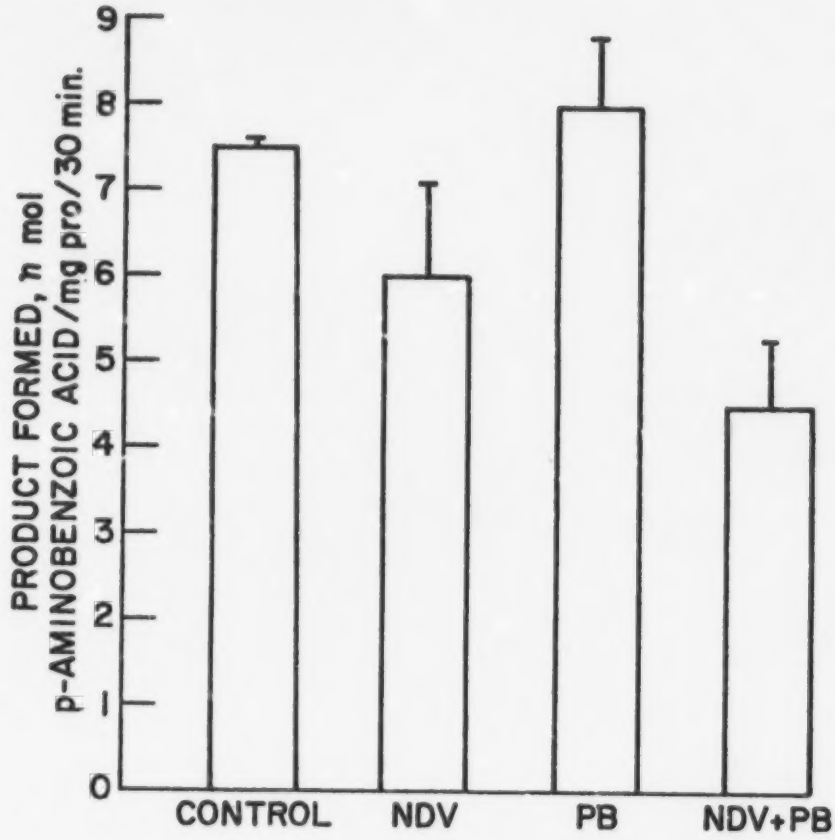

Figure 15. Effect of Newcastle disease vaccination on hepatic nitroreductase of eight-week-old pullets. NDV = Newcastle disease vaccinated group. PB = phenobarbital induced group. NDV + PB = group vaccinated with Newcastle disease virus and subsequently induced with phenobarbital. Data are mean activities ± std error of the mean for 5 chickens.

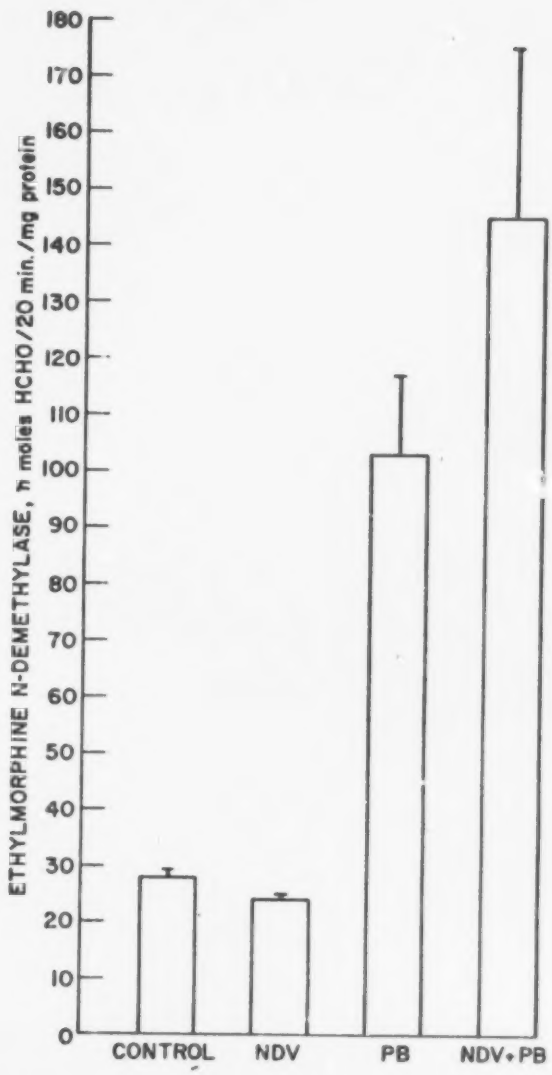

Figure 16. Effect of Newcastle disease vaccination on hepatic microsomal ethylmorphine N-demethylase of eight-week-old cockrels. NDV = Newcastle disease vaccinated group. PB = phenobarbital induced group. NDV + PB = group vaccinated with Newcastle disease virus and subsequently induced with phenobarbital. Data are mean activities ± std error of the mean for 5 chickens.

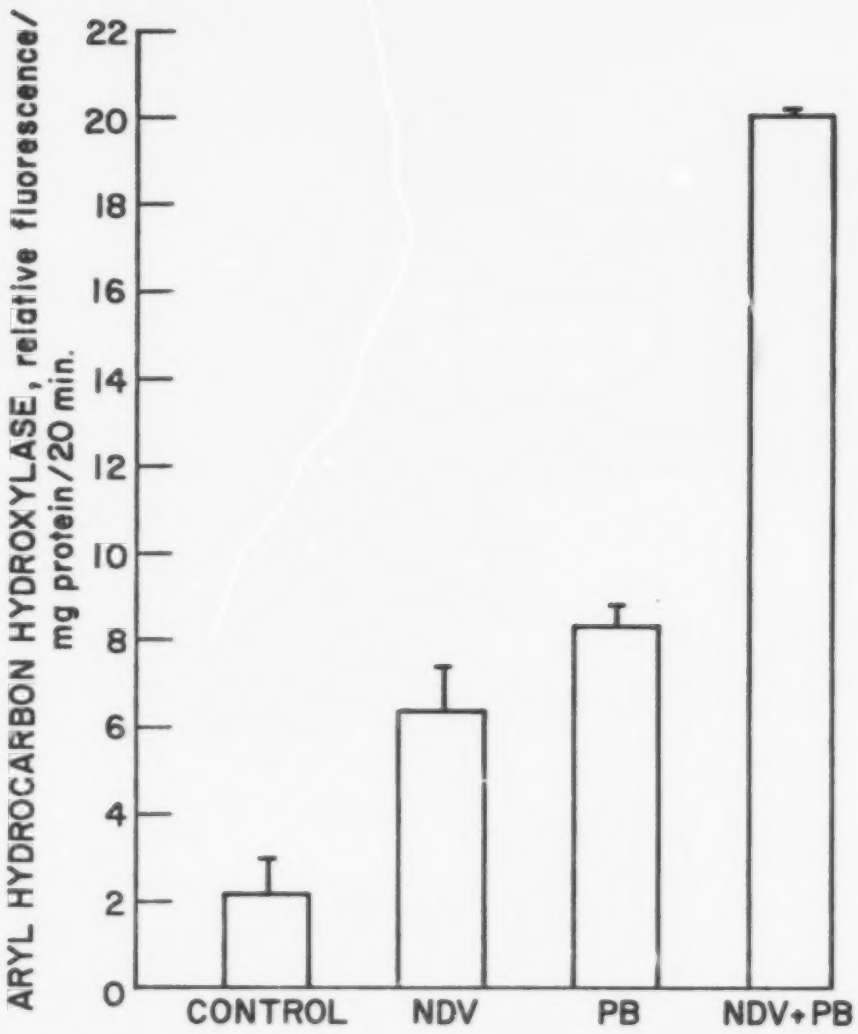

Figure 17. Effect of Newcastle disease vaccination on hepatic microsomal aryl hydrocarbon hydroxylase of eight-week-old cockrels. NVD = Newcastle disease vaccinated group. PB = phenobarbital induced group. NDV + PB = group vaccinated with Newcastle disease virus and subsequently induced with phenobarbital. Data are mean activities ± std error of the mean for 5 chickens.

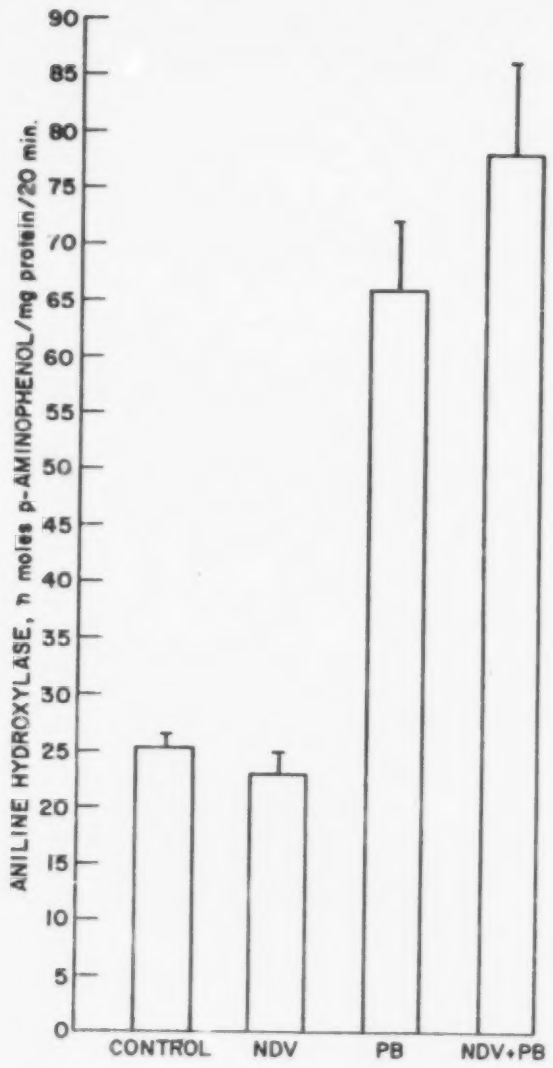

Figure 18. Effect of Newcastle disease vaccination on hepatic microsomal aniline hydroxylase of eight-week-old cockrels. NDV = Newcastle disease vaccinated group. PB = phenobarbital induced group. NDV + PB = group vaccinated with Newcastle disease virus and subsequently induced with phenobarbital. Data are mean activities ± std error of the mean for 5 chickens.

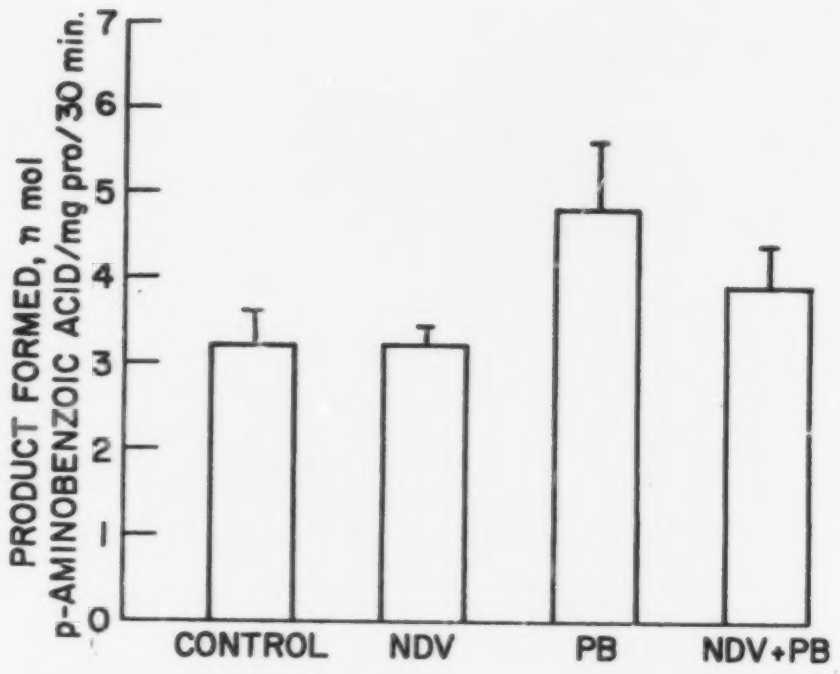

Figure 19. Effect of Newcastle disease vaccination on hepatic nitroreductase of eight-week-old cockrels. NDV = Newcastle disease vaccinated group. PB = phenobarbital induced group. NDV + PB = group vaccinated with Newcastle disease virus and subsequently induced with phenobarbital. Data are mean activities ± std error of the mean for 5 chickens.

SECTION VI

EFFECT OF DUCK HEPATITIS VIRUS ON THE INDUCTION OF DRUG-METABOLIZING ENZYMES IN DUCK LIVER AND ON TISSUE RESIDUE OF DDT.

Adult flighting mallard drakes were divided into two groups of 45 birds each. Two days prior to exposing the ducks to DDT, one group was injected intraperitoneally with $2 \times 10^{5.1}$ chick embryo LD_{50} of duck hepatitis virus and the other group was injected with an equal volume (0.2 ml) of 0.85% NaCl in 0.1 M sodium-potassium phosphate buffer, pH 7.4. The birds were fed a chick growing ration containing no feed additives ad libitum. On day "0" the birds were exposed to DDT by replacing the feed with the same feed to which p,p'-DDT (Aldrich Chemical Co., Milwaukee, WI) had been added with thorough mixing to a level of 900 ppm. The average intake of DDT was about 50 mg/kg/day. Just prior to the change in feed, all birds were weighed (bird weights ranged from 1.0 - 1.5 kg) and 5 from each group were killed. Blood serum, bile and brain were collected and stored at -26 C for pesticide residue analysis as described in Section IV. Liver was collected for enzyme analysis as described in Section IV. Five birds from each group were weighed, killed and the same samples taken for analysis on days 1,3,5,7,8,9 and 12.

Feed consumption was recorded (Fig. 20), however, because of the feeding habits of ducks this type of data is less reliable for ducks than for other species. Thus, the more pronounced variations must be considered lightly and the most reasonable conclusion is that there was no major difference between the virus-treated and control birds in the amount of feed eaten.

The body weights and liver weights at the time the ducks were killed are given in Figs. 21 and 22. Changes in body weight relative to the birds' weights at day 0 have been plotted in Fig. 23. The virus injected birds

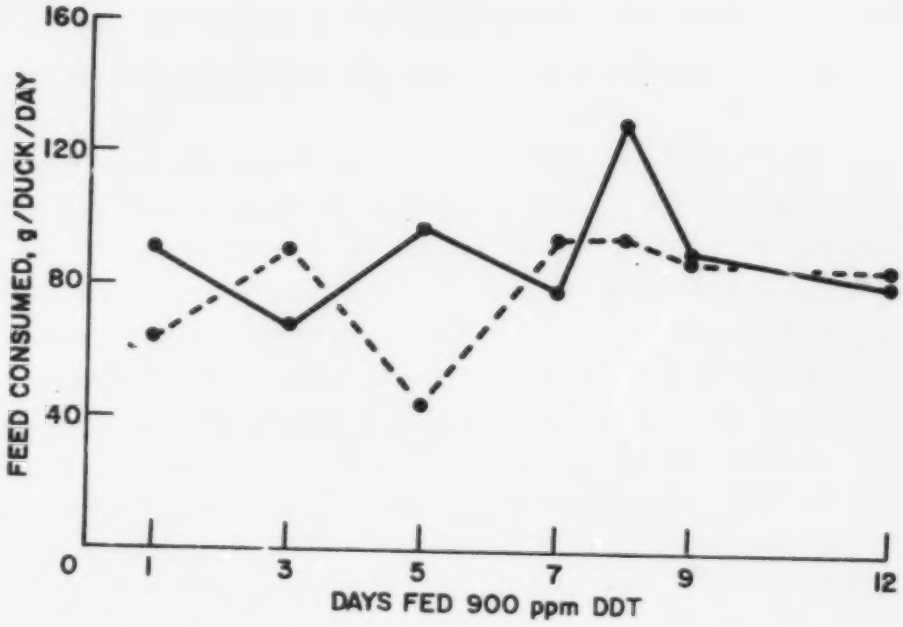

Figure 20. Feed consumption of DDT contaminated feed by ducks injected with duck hepatitis virus (broken line) or buffer (solid line).

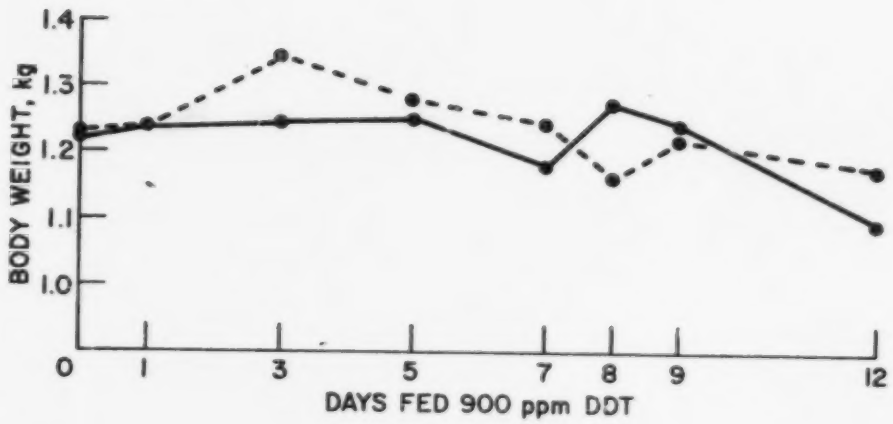

Figure 21. Body weights at time of killing of ducks injected with duck hepatitis virus (broken line) or buffer (solid line). Each point is the mean weight for 5 ducks.

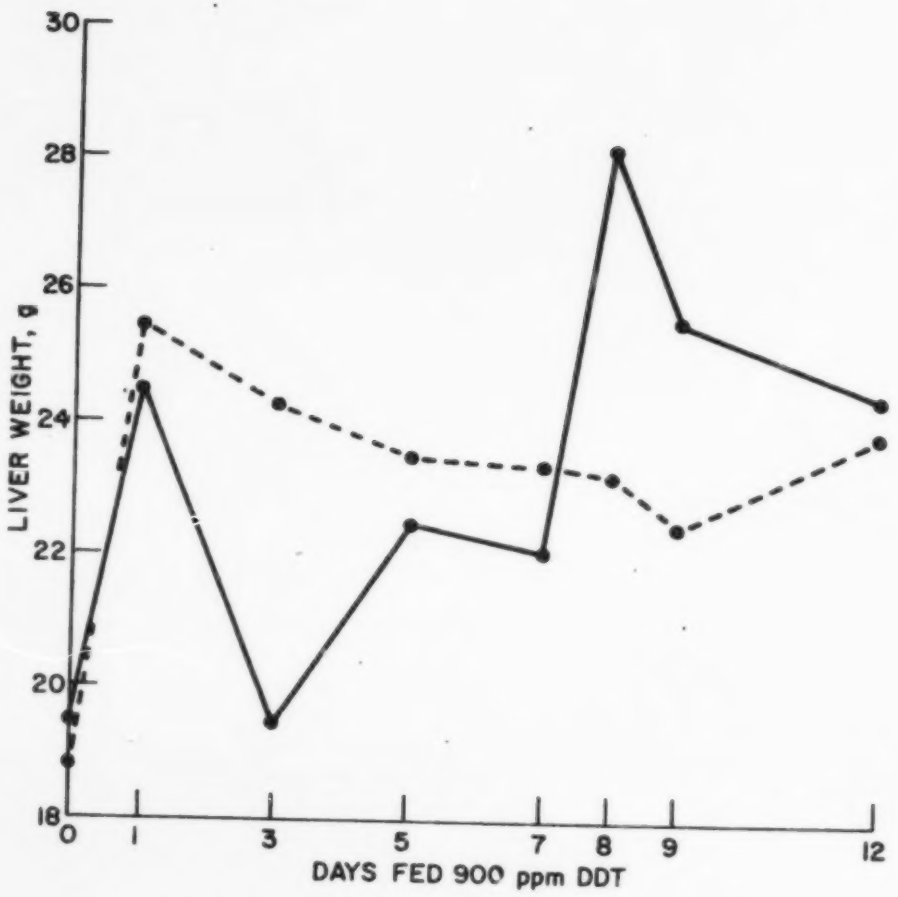

Figure 22. Liver weights at time of killing of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed 900 ppm DDT. Each point is the mean weight for 5 ducks.

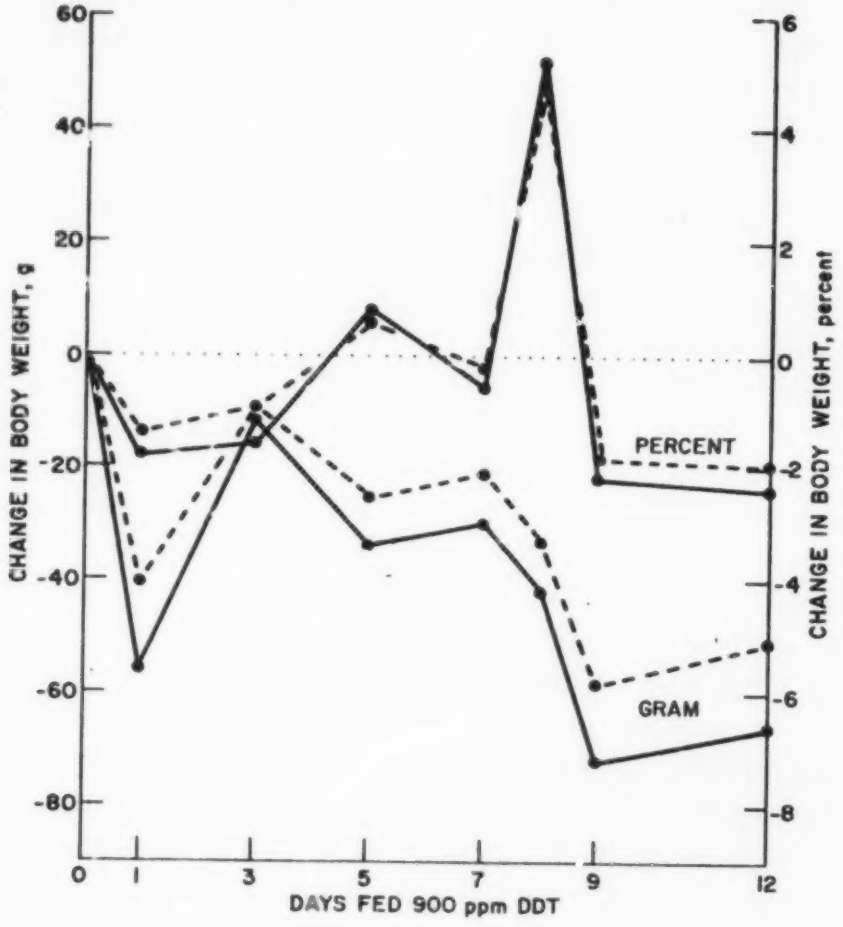

Figure 23. Changes in body weight during DDT treatment of ducks injected with duck hepatitis virus (broken line) or buffer (solid line). Each point is the mean value for 5 ducks.

lost on the average 45 gm or 3.5% of their body weight, whereas the control birds lost on the average 3.4 gm or 0.3% of their body weight. The virus injected birds killed on the last day had lost 5% of their body weight and the control birds killed on the last day had lost 2% of their body weight.

Increases in the amount of microsomes per gm liver (Fig. 24) and in the activities of the microscomal drug-metabolizing enzymes (Figs. 25-27) both provide evidence of induction by DDT. Microsomal content did not increase as rapidly in the livers of ducks injected with duck hepatitis virus until about the eighth day when the rate of increase exceeded that of the control birds.

The differences in microsomal content are not reflected in the enzyme data in Figs. 25-27 since the data is recorded on the basis of microsomal protein The activities of all 3 enzymes were greater in the virus-treated birds for the first 3 days with one exception and that was N-demethylase which was lower on day 1 ($p < 0.05$). On day 5, however, the activities of all 3 enzymes were lower in the virus-treated birds, but on days 7 and 8 were again higher than those of control birds. The point of greatest statistical significance was day 8 when all three enzyme activities were greater in the virus-treated birds at the 0.05 to 0.01 level. On day 9 the activities had fallen to the same levels as the control birds but they had increased again by day 12.

The results of pesticide residue analysis are given in Figs. 28-34. The total pesticide residue (DDT + DDD + DDE) in serum, bile and brain is given in Fig. 28. Total pesticide concentrations in serum and brain accumulated throughout the course of the experiment. Concentration of total pesticide in the bile increased for 4-5 days after which it remained relatively constant. Although total pesticide continued to accumulate in serum, most of it was non-metabolized DDT with only slight increases in DDE and DDD (Fig. 29). The relative concentrations of DDD in serum decreased from 12 and 18% to 8% of total pesticide and the relative concentration of DDE decreased from 22 and 28% to 14% of the total pesticide.

In bile, the level of DDT increased for 3-4 days after which it remained relatively constant (Fig. 30). There was no difference between experimental and control groups. There also was no difference between DDE levels

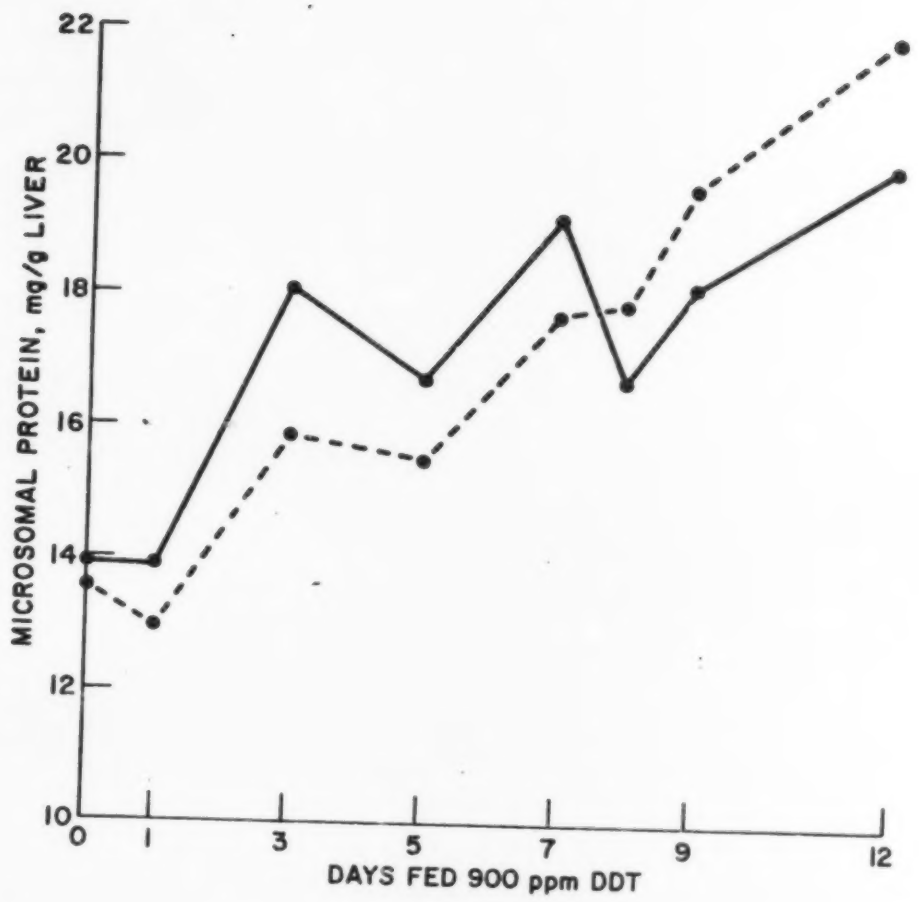

Figure 24. Amount of microsomes per gm duck liver with time fed 900 ppm DDT. Broken line = ducks injected with duck hepatitis virus. Solid line = ducks injected with buffer. Each point is the mean value for 5 ducks.

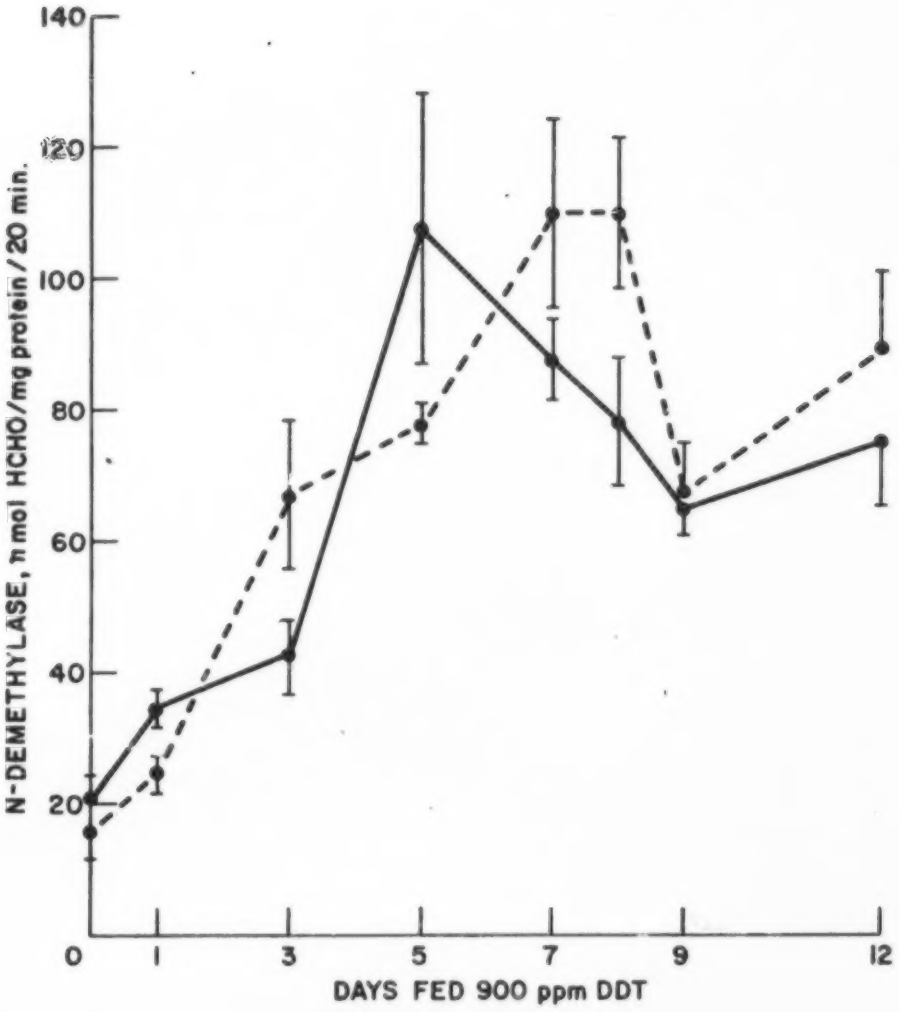

Figure 25. Activities of ethylmorphine N-demethylase in livers of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed 900 ppm DDT for 12 days. Each point is the mean activity ± std error of mean for 5 ducks.

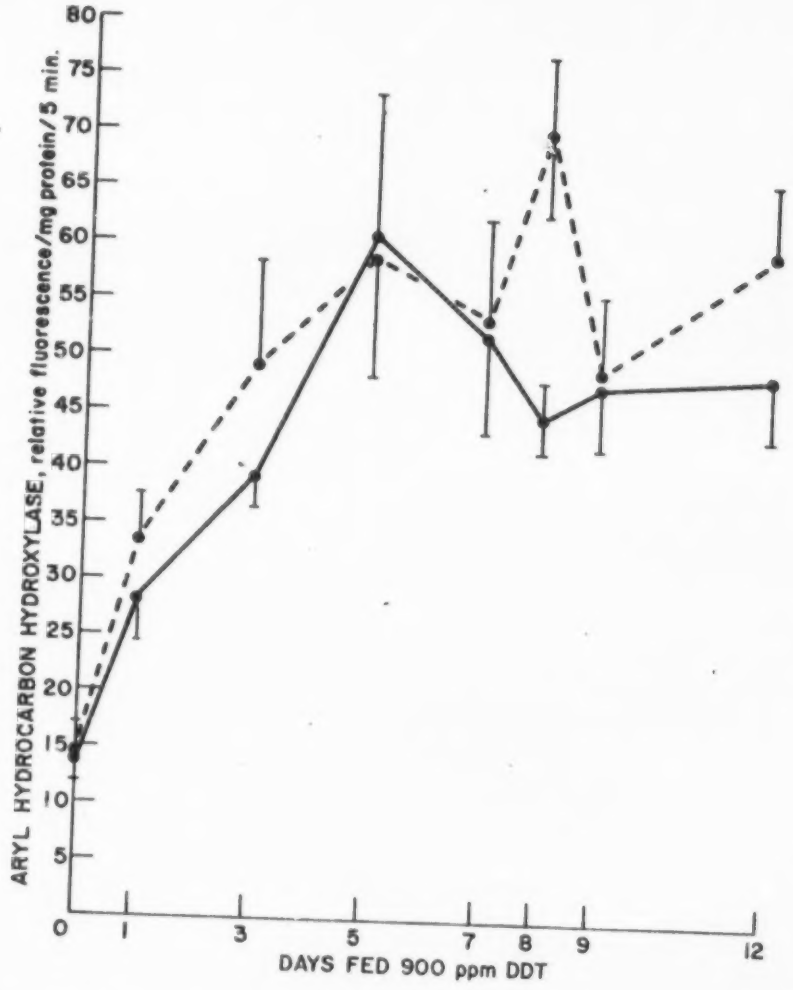

Figure 26. Activities of aryl hydrocarbon hydroxylase in livers of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed 900 pmm DDT for 12 days. Each point is the mean activity ± std error of mean for 5 ducks.

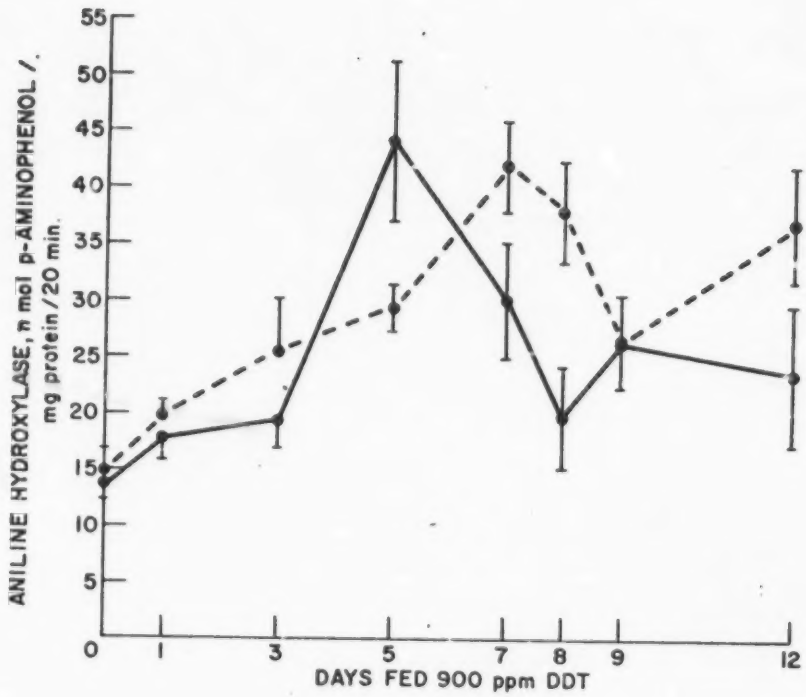

Figure 27. Activities of aniline hydroxylase in livers of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed 900 ppm DDT for 12 days. Each point is the mean activity ± std error of mean for 5 ducks.

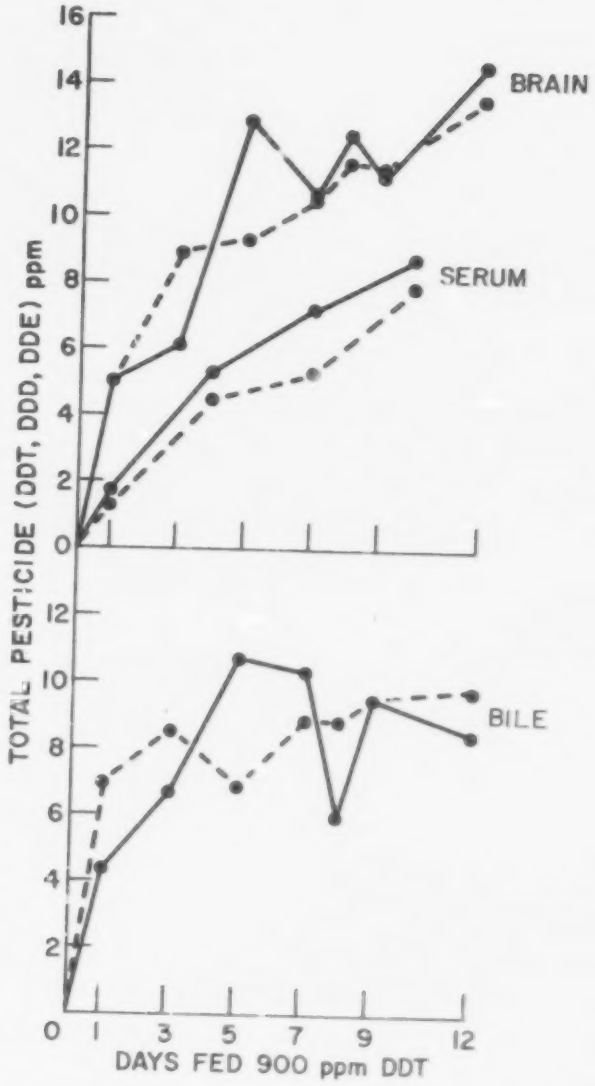

Figure 28. Total pesticide residue (DDT + DDD + DDE) in serum, bile and brain of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed DDT for 12 days. Each point is mean value for 5 ducks.

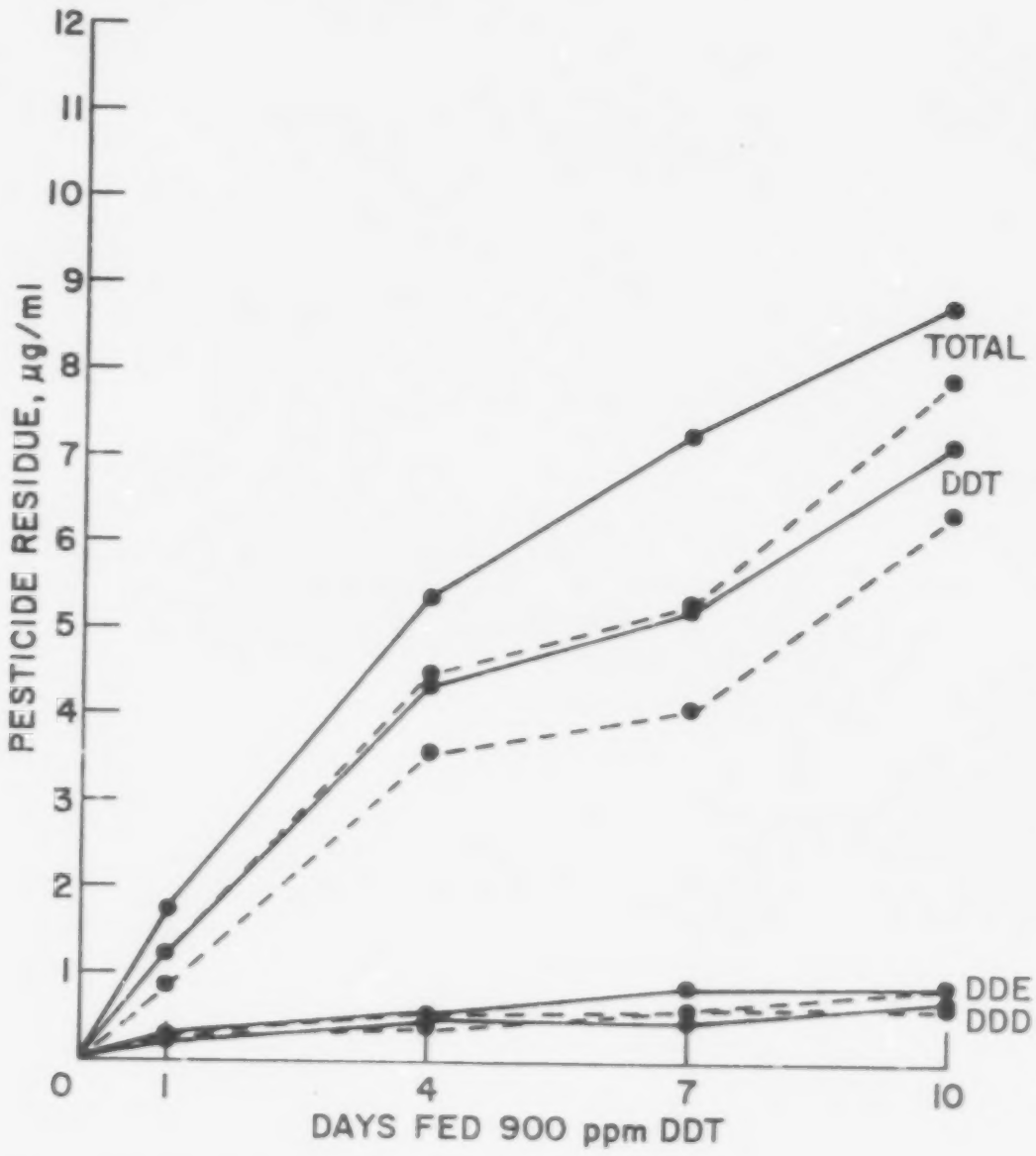

Figure 29. Pesticide levels in serum of ducks injected with duck hepatitis virus (broken line) of buffer (solid line) and fed DDT for 10 days. Each point is mean value for 5 ducks.

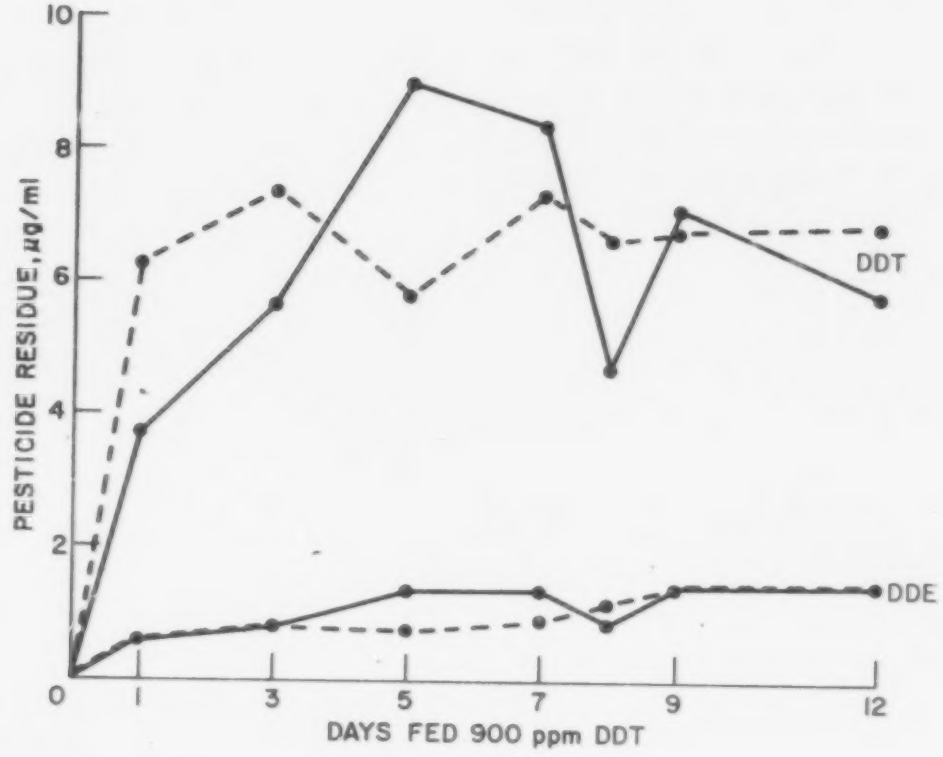

Figure 30. Concentration of DDT and DDE in bile of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed DDT for 12 days. Each point is mean value for 5 ducks.

in the two groups (Fig. 30). The rate of increase leveled off after several days but appeared to continue to rise slowly which is more evident in Fig. 31. The level of DDD increased continually and was consistently greater in the experimental group after 7 days and consistently greater after 3 days when expressed as percent of total pesticide (Fig. 32). The ratio of DDD to DDE in bile was greater in the experimental group from the first day (Fig. 33).

The residues of DDT, DDD, and DDE in brain are given in Fig. 34. The concentrations of all 3 residues continued to accumulate. The relative concentration of DDE increased slightly from 10% of total at day 1 to 15-21% on day 12. The relative concentration of DDD remained constant at about 5% of total.

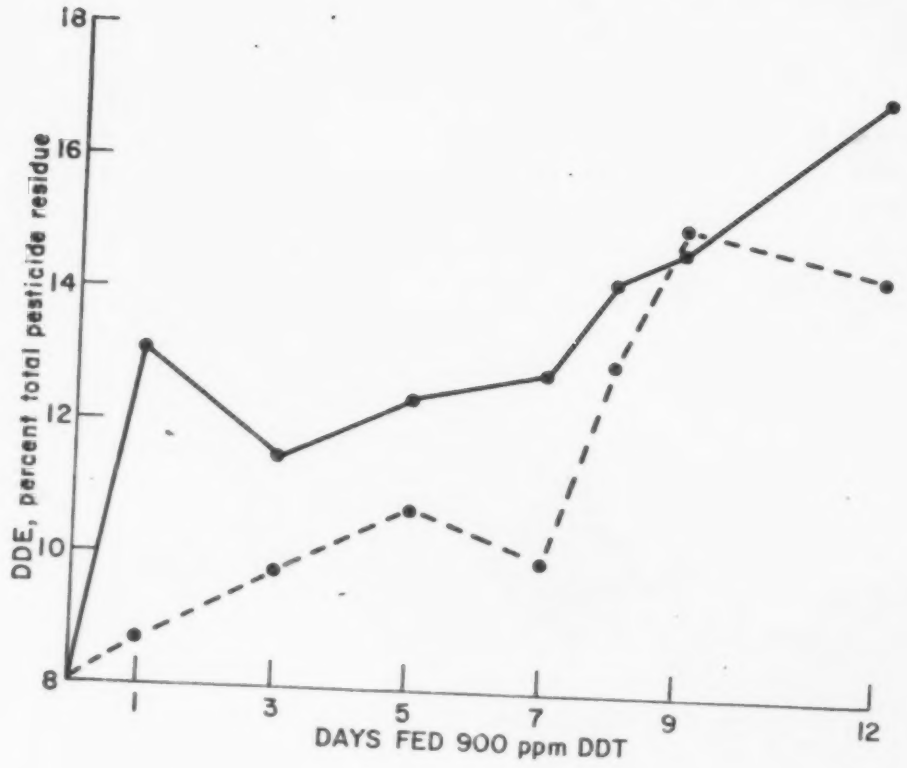

Figure 31. Concentration of DDE relative to total pesticide in bile of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed DDT for 12 days. Each point is mean value for 5 ducks.

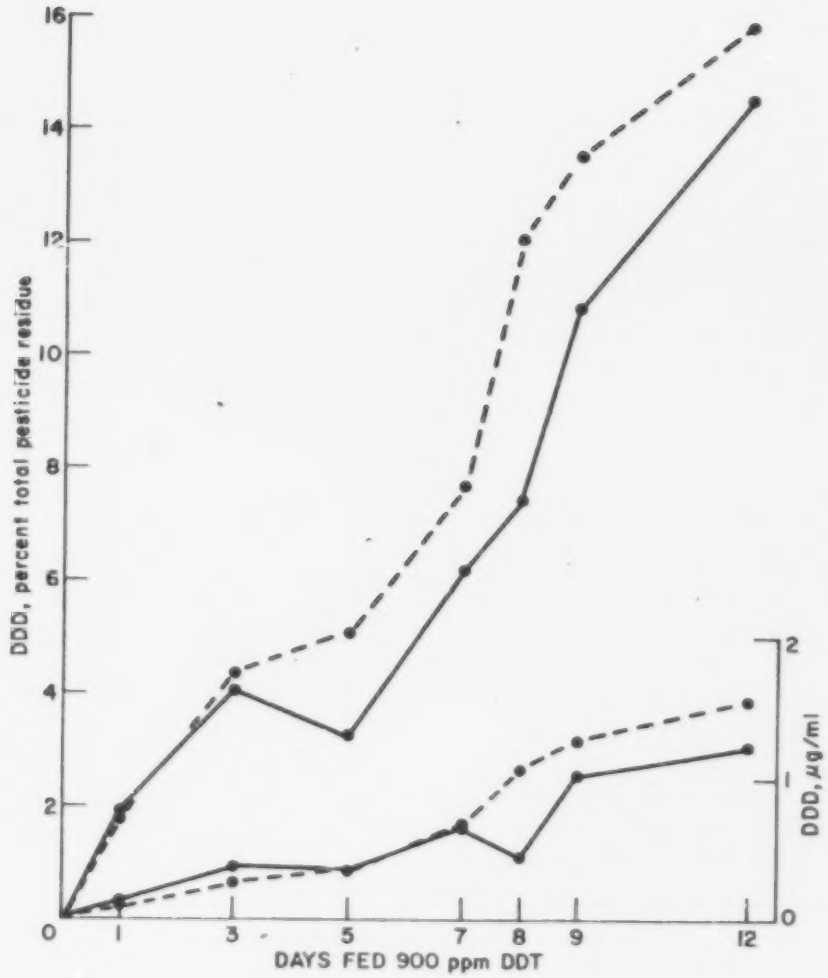

Figure 32. Concentration of DDD and concentration of DDD relative to total pesticide in bile of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed DDT for 12 days. Each point is mean value for 5 ducks.

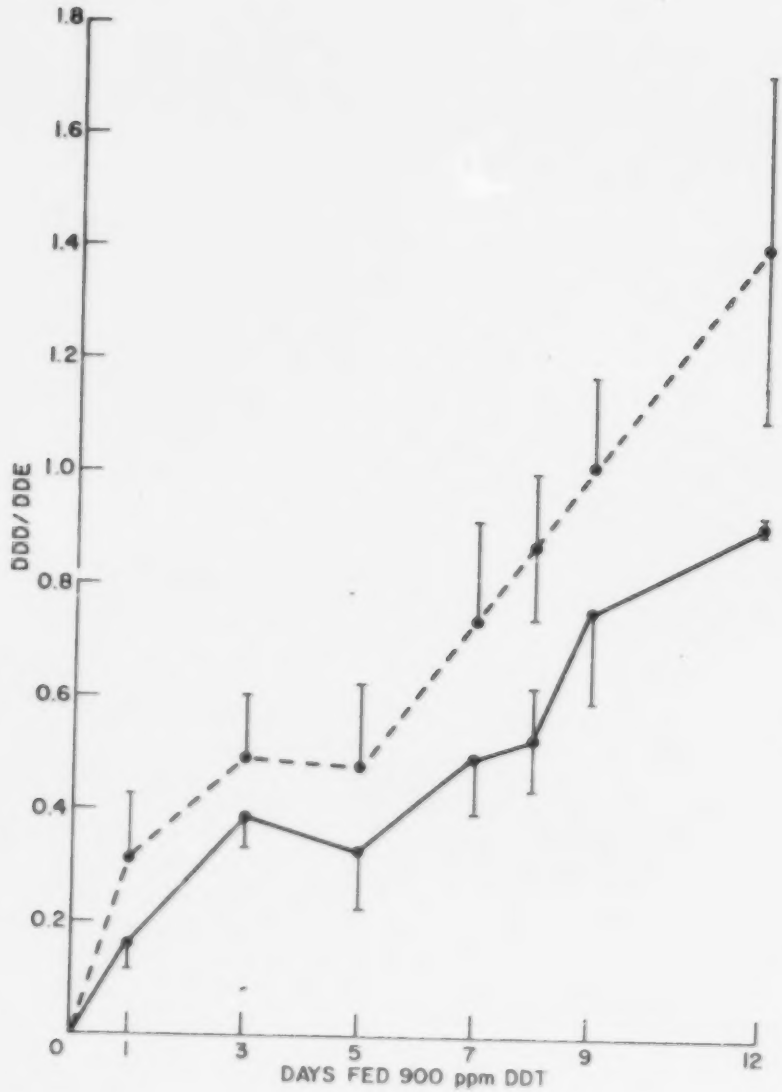

Figure 33. Ratio of DDD to DDE concentrations in bile of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed DDT for 12 days. Each point is mean value ± std error of mean for 5 ducks.

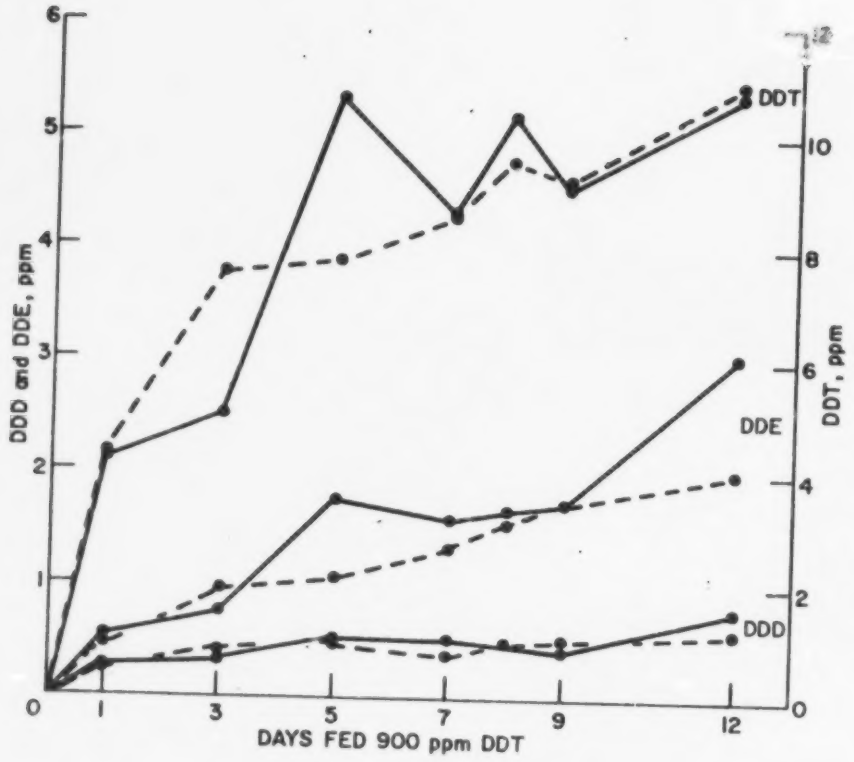

Figure 34. Concentration of DDT, DDD and DDE residues in brains of ducks injected with duck hepatitis virus (broken line) or buffer (solid line) and fed DDT for 12 days. Each point is mean value for 5 ducks.

SECTION VII

EFFECT OF NEWCASTLE DISEASE VACCINAL VIRUS ON THE INDUCTION OF DRUG-METABOLIZING ENZYMES AND ON TISSUE RESIDUE OF DIELDRIN IN CHICKENS.

Commercial broiler type pullets which had been accidentally contaminated with dieldrin were obtained in April, 1974 from McCarty Enterprises, McGee, Mississippi. The birds were offered to us with the hope that vaccination with Newcastle disease vaccinal virus would provide a means of accelerating the excretion of tissue residues. The birds had been fed the contaminated diet from 1 to 19 days of age when the feed was changed to an unadulterated ration. Five birds had been killed at 19, 24 and 30 days of age and body fat assayed for dieldrin content by Woodsen-Tenent Co. of Memphis, Tennessee. The average dieldrin residues were 1.20, 0.80, and 0.95 ppm respectively.

Five birds were killed at 52 days of age and abdominal fat samples assayed for dieldrin as described in Section IV. Twenty birds were separated into 4 groups of 5 birds each. The birds in two groups were injected intraperitoneally with 5×10^6 chick embryo LD_{50} of the LaSota B-1 strain of Newcastle disease virus. Three days later and for 2 additional days the birds in one of the virus groups were injected intraperitoneally with 40 mg sodium phenobarbital in sterile saline / kg body weight and the birds in the other group were injected with equivalent volumes of sterile saline. The birds in the two non-virus groups were injected on the first day with sterile diluent and the birds in one group were treated with phenobarbital on the third through the fifth day while the other group was injected with sterile saline. Birds in all four groups were killed on the 7th day and

samples taken for residue analysis and assay of hepatic microsomal ethylmorphine N-demethylase and aryl hydrocarbon hydroxylase.

Vaccination resulted in increased activities of both microsomal enzymes but they were not increased as much by vaccination as they were by phenobarbital (Fig. 35). In contrast with the results recorded in Sections V and VI, viral replication followed by phenobarbital treatment did not produce any greater increase in enzyme activities than with phenobarbital treatment.

Dieldrin residues (Table 1) were essentially the same in all of the birds regardless of treatment.

Table 1. CONCENTRATIONS OF DIELDRIN IN ABDOMINAL FAT OF CHICKENS ACCIDENTALLY CONTAMINATED WITH DIELDRIN

Treatment group	Day killed	Concentration, ppm[a]
Untreated	0	0.24 ± 0.03
Saline only	21	0.11 ± 0.01
NDV	21	0.14 ± 0.01
Phenobarbital	21	0.15 ± 0.03
NDV + Phenobarbital	21	0.14 ± 0.01

[a] Mean ± standard error of the mean for 5 ducks.

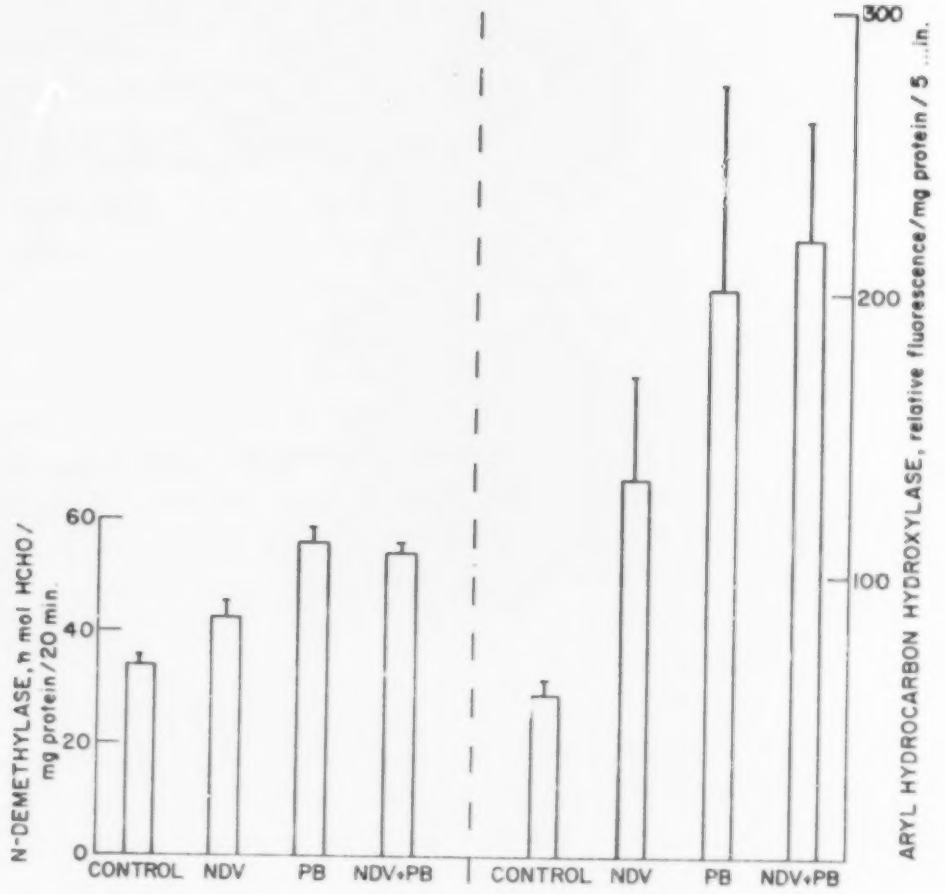

Figure 35. Ethylmorphine N-demethylase and aryl hydrocarbon hydroxylase activities of hepatic microsomes from dieldrin-contaminated chickens treated with Newcastle disease vaccinal virus and phenobarbital. Data are mean activities ± std error of mean for 5 chickens.

SECTION VIII

DISCUSSION

Duck hepatitis viral replication in the livers of adult ducks causes specific, subtle changes in the hepatic microsomal drug-metabolizing enzymes. Previously reported results were confirmed in the present study. Not only does duck hepatitis virus produce these changes in duck liver but Newcastle disease vaccinal virus produces similar changes in chicken liver. It now appears that, when the reported effects of murine hepatitis virus[3] and human hepatitis virus type B[6] are taken into consideration, specific effects on microsomal drug-metabolizing enzymes may be characteristic of many viruses which produce hepatitis. Of even greater interest (and importance) is the observation that viruses such as Newcastle disease vaccinal virus which replicate on hepatic endoplasmic reticulum without causing hepatic disease may also specifically alter the hepatic drug-metabolizing enzymes.

Replication of Newcastle disease virus in the absence of a chemical inducer did not result in any consistent increases in enzyme activities. Although the results were variable and occasionally the microsomal enzyme activities were slightly higher than controls, the trend was to activities slightly lower than controls similar to the effects of duck hepatitis virus in ducks.[5] Although the replication of Newcastle disease virus in dieldrin-contaminated chickens (not subsequently induced with phenobarbital) resulted in higher enzyme activities than in the control birds (Fig. 35), none of the birds can be considered naive in regard to chemical induction. All had been exposed to dieldrin and still carried significant pesticide residues in their tissues. Although the activities of the two microsomal enzymes were well below the maximum inducible levels and were surely

in the process of returning from higher to normal levels, they still were well above the levels of normal birds. Thus, it must be considered that the birds' microsomal enzymes were still responding to the chemical inducer, dieldrin, or specific changes in the endoplasmic reticulum produced in response to dieldrin were still present and, thus, the higher enzyme activities from viral replication were due to an additive effect not possible in naive birds.

Although the levels of the drug-metabolizing enzymes in chickens were not increased by viral replication in the absence of a chemical inducer (phenobarbital), the activities of the microsomal enzymes were considerably greater in the vaccinated birds given phenobarbital than in nonvaccinated birds given phenobarbital. This "superinduction" or "facilitation of induction" was statistically significant in the case of aryl hydrocarbon hydroxylase in both males and females. The lack of statistical significance at the 5% level in the case of the other two enzymes is due in part to the relatively small sample size. The percent increase is impressive, however, particularly for N-demethylase, and implies biological significance. Furthermore, the inductive or "facilitative" effect of vaccination probably was more striking in the case of aryl hydrocarbon hydroxylase because phenobarbital is a weak inducer of this enzyme. Since phenobarbital at the dosage used in this study is a strong inducer of N-demethylase and aniline hydroxylase, the levels of these enzymes were already induced to such high levels by phenobarbital in the nonvaccinated birds that it would be difficult to demonstrate statistically significant differences between the phenobarbital group and the "superinduced" levels in the vaccinated group. A weak chemical inducer of these enzymes would have to be used in order to establish statistically significant differences by viral "superinduction" (viral "facilitation" of induction may be a better term since viral replication in the absence of a chemical inducer does not produce increased enzyme activity).

The activities of the soluble enzyme, nitroreductase, were not increased significantly by phenobarbital, virus or virus plus phenobarbital. The activities in the virus plus phenobarbital group were lower than the phenobarbital group rather than higher. The reason for the decreased activity is not apparent.

In the duck experiments, the three microsomal enzymes were significantly higher in the virus treated group than in the non-virus group on the 8th day. Aniline hydroxylase and N-demethylase were also higher on the 7th day and the data appear to be consistent with the previously reported higher activities on the 7th day.[5] In the preliminary work several years ago, increases were also noticed on the 3rd day (Ragland et al., unpublished observations) but data were not collected on the 3rd day in a subsequent experiment which served as the basis for the published report. It is interesting to observe that the microsomal enzymes were also increased on the 3rd day in the present study, thus, confirming the previously observed increases on days 3 and 7. The enzyme activities at other intervals of time were altogether different and not at all what had been anticipated on the basis of previous results. Nevertheless, since increased activities have been observed in the virus group on day 3 on two occasions and on day 7 or 8 on three occasions, we can be fairly confident that facilitation of enzyme induction by virus occurs at these times. The activities of the three enzymes were higher in the virus group on days 3 and 7-8, and on day 12 they appeared to be on the rise again. On days 1, 5 and 9 the activities in the virus group had fallen to or below the levels of the control group (that is, the chemically induced group). It was as if the rise and fall in enzyme activity in the virus group is on some schedule regulated by the viral replicatory cycle. This is an intriguing speculation but at the present time must remain that - speculation.

The observed effects of viral replication on enzyme activities or pesticide residues cannot be attributed to generalized marasmic consequences of viral replication. There was no major difference in feed consumption by the two groups and no striking differences in body weight. Although the losses in body weight would have been costly to a commercial grower the losses were not severe enough to cause profound biological consequences in the birds. The variation in liver weight was greater but the analytical data cannot be ascribed to these differences. The results cannot be explained on the basis of differences in body weight or liver weight, as the ratios of the virus group to the control group at days 3 and 8 are reversed and thus would be contradictory. Finally, the general health of the birds could not have been greatly impaired as the livers of birds in both groups

were able to increase their microsomal content (Fig. 24). Although the birds in the virus group were initially less efficient in responding to the DDT by increasing the synthesis of endoplasmic reticulum, the cause was a specific viral effect rather than generalized debilitation as evidenced by greater synthesis of endoplasmic reticulum in the virus group later on.

The total pesticide in brain and serum continued to increase throughout the experiment. Total pesticide in bile leveled off by the 5th day (Fig. 28) indicating that the livers were handling as much pesticide as they could. That the ducks were taking in more pesticide than they could handle is substantiated further by the fact that most of the pesticide in brain and in serum was DDT. Conversion of DDT to DDD and DDE was stimulated and both metabolites accumulated in brain and serum at approximately the same rate.

Excretion of DDD and DDE in bile was different. Although both were excreted in bile in increasing amounts, the excretion of DDD was progressively greater than DDE throughout the experiment (Fig. 33). The greatest significant difference was only at the 10% level, but if there were no difference between virus and control groups, the chance that DDD excretion would be greater at every interval examined is 2^7, or one chance in 128. The results are consistent with the hypothesis that viral replication on endoplasmic reticulum alters the microsomal enzymes specifically. The enzymes which convert DDT to DDD are microsomal[24] whereas DDT is converted to DDE by a soluble enzyme.

According to Hill *et al.*[25] ca. 20 ppm DDD + DDT in brain is a lethal concentration and ca. 10 ppm is associated with clinical signs of intoxication. The brains of ducks in this study contained up to 11.5 ppm which was associated with mild hyperexcitability and there were no deaths.

Friend[4] reported that the total pesticide residue in brains of ducks treated with virus prior to feeding DDT were markedly lower than in untreated birds fed DDT. Only several brains were assayed, however, and we have been unable to confirm his observation in this larger study. It is therefore questionable at the present time that the resistance to DDT toxicity afforded to ducks by prior exposure to duck hepatitis virus[4,5] is due

to enhanced detoxification by induced microsomal enzymes even though the enzyme activities were elevated at several intervals following exposure to the virus. In addition, vaccination of dieldrin-contaminated chickens for Newcastle disease failed to effect increased clearance of the pesticide even though the microsomal enzymes had been induced. The pesticide load in the chickens had decreased to such a low level before vaccination that the conditions under which the experiment was conducted were not ideal and probably were inadequate for a proper test of the efficacy of viral replication to increase clearance.

SECTION IX

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SECTION X

LIST OF INVENTIONS

1. No inventions.
2. The following abstract of a paper presented at the fourteenth annual meeting of The Society of Toxicology, Williamsburg, Virginia, March 9-13, 1975, will be published in the Society's journal.

   Ragland, W. L. and S. J. Buynitzky. Effects of Viral Replication on Drug Metabolism. Toxicology and Applied Pharmacology, in press.